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Analysis of the specification of the abdominal leucokinergic neurons in the central nervous system of *Drosophila melanogaster*

DOCTORAL THESIS

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Analysis of the specification of the abdominal leucokinergic neurons in the central nervous system of *Drosophila melanogaster.*

Doctoral thesis presented by Alicia Estacio Gómez to fulfill the requirements for the degree of Doctor in Molecular Biology, Universidad Autónoma de Madrid.

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“Any man could, if he were so inclined, be the sculptor of his own brain”.

Advice for a Young Investigator.

Santiago Ramón y Cajal.

The present work has been performed in the laboratory of Dr. Fernando Jiménez Díaz-Benjumea, in the Institute of Molecular Biology Severo Ochoa (CSIC-UAM), with the support of a postgraduate studies grant by Universidad Autónoma de Madrid, a pre-doctoral fellowship from the Spanish Ministry of Education (AP2008-00397), and a contract from the Research Project "Especificación de destinos neurales en el sistema nervioso central de *Drosophila*", granted by the Ministry of Science and Innovation (BFU2011-24315).

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1. Summary

The central nervous system (CNS) exhibits by far more cellular diversity than any other organ, and it has become an excellent model to study cell fate specification. However, little is known about the specification of a particular set of neurons. Here, we have focused on the study of the mechanisms underlying the specification of the abdominal leucokinergic neurons (ABLKs), located in the ventral nerve cord of *Drosophila* CNS and characterized by the expression of the neuropeptide Leucokinin (Lk). They form a group of 14 cells, one per hemineuromere, in the abdominal segments (A1-7).

First, by a combination of different molecular markers and lineage tracing experiments, we have identified neuroblast 5-5A as the progenitor which gives rise to ABLKs. Besides, we have found that these neurons are specified in a Castor/Grainy-head (Cas/Grh) temporal window.

Second, we found that both postmitotic cells, the ABLK and its sibling cell, are equally competent to activate Lk expression, but are fated to die unless Notch signalling gets activated in one of them and represses the apoptotic programme.

Third, we performed a genetic screen to identify genes involved in ABLKs specification. We found *nab, klumpfuss (klu), squeeze (sqz), jumeaux, knirps* and *teashirt*.

We have also observed that the number of ABLKs increased during pupal stages, reaching an average of 10 in four-day-old adults. By lineage-tracing experiments, we concluded that the ABLKs observed in adults corresponded to the embryonic ABLKs (eABLKs) plus the additional ones that were generated during the larval neurogenesis; we named them postembryonic ABLKs (pABLKs). Then, we discovered that pABLKs came from the ventrolateral postembryonic NB (vl pNB) and concluded that the embryonic NB5-5A and the vl pNB were the very same neuroblast.

Lastly, we studied the role of *Hox* genes in the segment-specific appearance of ABLKs, and found that Ultrabithorax and Abdominal-A are redundantly required to specify them; and that Abdominal-B represses the expression of Lk in abdominal segment 8 during embryogenesis, whereas it does so in A5-8 during the postembryonic neurogenesis.

2. Abbreviations

ABLKs: abdominal leucokinergic neurons.

AEL: after egg laying.

ALKs: anterior leucokinergic neurons.

AP: anteroposterior.

BBT: PBT with 0.1% BSA and 250 mM NaCl.

bHLH: basic helix-loop-helix.

BSA: bovine serum albumin.

CCAP: Crustacean cardioactive peptide.

CNS: central nervous system.

CPN: cortical projection neuron.

DV: dorsoventral.

eABLKs: embryonic ABLKs.

eNB: embryonic neuroblast.

FMRFa: FMRFamide.

GFP: green fluorescent protein.

GMC: ganglion mother cell.

IPC: intermediate progenitor cell.

LHLKs: lateral horn leucokinergic neurons.

Lk: leucokinin.

NB: neuroblast.

N signalling pathway: Notch signalling pathway.

pABLKs: postembryonic ABLKs.

PBS: Phosphate buffer solution.

PBT: PBS with 0.3% Tween 20.

PCD: programmed cell death.

PLC: peptidergic lateral cluster.

pNB: postembryonic neuroblast.

RGC: radial glial cell.

RT: room temperature.

SELKs: subesophageal leucokinergic neurons.

SNa: segmental nerve a.

SVZ: subventricular zone.

TN: transverse nerve.

UAS: upstream activation sequence.

VNC: ventral nerve cord.

VZ: ventricular zone.

3. Introduction

Developmental biology is the study of the process by which an organism grows and develops, from a single cell into an adult. Thus, it includes the study of cell proliferation, differentiation and morphogenesis. However, as most multicellular organisms keep, in a way, developing in adults stages, it also encompasses the study of regeneration and aging. In essence, the fundamental questions in developmental biology are to understand how an specific system is functionally set up, how cells manage to integrate the numerous signals they receive and how they are able to create such cellular diversity from a somewhat small and homogeneous set of progenitor cells. There are few areas of developmental biology in which the address of these questions is as challenging as in the study of the central nervous system (CNS).

The CNS of higher organisms is unique in its complexity of cell types, highly precise cell connectivity, and exquisite sensitivity to environmental inputs (Pearson and Doe, 2004). In particular, the human CNS contains a daunting number of cells ($>10^{11}$) that comprise, at least, 10,000 different cell types of neurons and glia.

Remarkable progress has been made in many aspects of nervous system development including acquisition of neural competence and establishment of spatial and temporal cues that regulate the properties of progenitors and their progeny (Reviewed in (Jessell, 2000; Skeath and Thor, 2003)). However, there are still unanswerered issues about how this vast cellular diversity is achieved from a seemingly uniform and relatively small pool of progenitor cells. Work during the last two decades have revealed that neuronal cell fate is not dictated by the action of a single regulatory gene, but rather by the sequential and combinatorial action of many regulators and their unique interplay with key signalling pathways (Shirasaki and Pfaff, 2002). In fact, the appearance of the proper combinatorial code in a neuron is the final result of a sequence of earlier and increasingly restricted regulatory events (Jessell, 2000).

The aim of this thesis is to dissect the genetic and molecular mechanisms underlying neural specification during the development of the CNS. For this purpose, due to its amenability and the extensive genetic modifications that it allows, we have chosen *Drosophila melanogaster* CNS as a model system, and the study of the specfication of a group of neurons that express the neuropeptide leucokinin (Lk) in the most anterior abdominal segments.

3.1. The CNS of arthropods and chordates share a common origin

Both arthropods and chordates possess a centralized nervous system. This consists of a brain and spinal cord, and it is present in most bilateral animals (Arendt et al., 2008).

Bilateral animals are classified into two large groups: Gastroneuralia and Notoneuralia (Nielsen, 1995). Gastroneuralia organisms have a ventral nerve cord. This group includes annelids and arthropods (*Drosophila*, among others). Notoneuralia animals have a dorsal nerve cord and include chordates (vertebrates). This opposing localization of the CNS posed whether the CNS of vertebrates and invertebrates had a common CNS precursor or whether they had an independent origin. Despite this difference, several evidence suggest that the molecular mechanisms involved in both neurogeneses are similar (Arendt and Nubler-Jung, 1999).

Both insects and vertebrates divide their ectoderms into a neurogenic and non-neurogenic region by the opposing activities of *decapentaplegic* (*dpp*; vertebrate Bone morphogenetic protein 4, *BMP4*) and *short gastrulation* (sog; vertebrate *chordin, chd*). In *Drosophila*, BMP signaling is antagonized by Dorsal, whereas in vertebrates Sonic Hedgehog (Shh) does so ventrally. In the two groups, the prospective nervous systems consist of a neuroepithelium that will later generate progenitor cells, most of which are multipotent and self-renewable. In both cases the progeny is generated towards the body cavity, producing different layers of neural cells according to their birth date. However, due to vertebrates neurulation, the innermost layer of neurons in insects corresponds to the outermost layer in vertebrates. Most importantly, in vertebrates as well in flies, orthologues sets of genes regionalize their neuroectoderms and are expressed in comparable areas and perform similar functions. In the anteroposterior (AP) axis, anterior brain parts express *orthodenticle* (*otd*; vertebrate *Otx-1/Otx-2*) among other genes, whereas posterior regions express *Hox* genes. In the dorsoventral (DV) axis, the nervous system is divided into columns by the expression of *ventral nervous system defective (vnd;* vertebrate *NK2 transcription related locus 2, Nkx2.2), intermediate neuroblasts defective (ind;* vertebrate *Genomic screen homeobox 1, Gsh-1)* and *muscle segment homeodomain (msh;* vertebrate *Msx-1/2/3).* (Fig. I.1 extracted from (Arendt and Nubler-Jung, 1999).

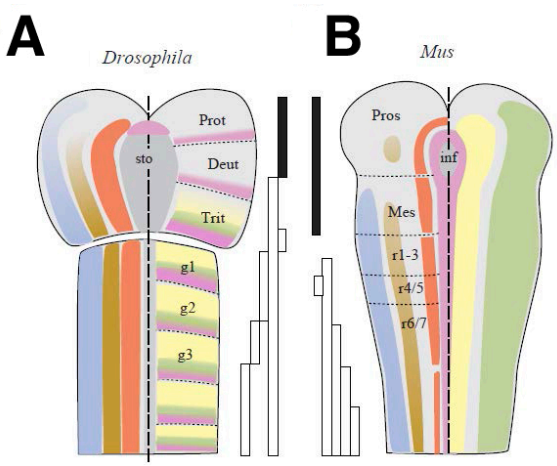


Fig. I.1. The CNS of arthropods and chordates share a common origin. Expression of *msh/Msx* (blue), *ind/Gsh-1* (brown), *vnd/Nkx2.2* (red), *hedgehog/Shh* (violet), *gooseberry/Pax-3/7* (green), patched (yellow), *orthodenticle/Otx* (black bars) and *Hox* homologues in the neuroectoderm of (**A**) *Drosophila* and (**B**) mouse at early stages of neural development (with the neural tube infolded into a neural plate for better comparison). (Arendt and Nubler-Jung, 1999)

Therefore, most evidence suggest that *Drosophila* and vertebrates nervous systems share a common ancestor that already possessed many of the characteristics that today arthopods and chordates have. Thus, it is generally assumed that the CNS of these two groups of animals are homologous structures and not the result of convergent evolution. So, the ventral nerve cord of insects may correspond to the dorsal nerve cord of vertebrates, and their presence in opposing sides in the present was due to an inversion of the dorsoventral axis during animal evolution (Arendt and Nubler-Jung, 1996; De Robertis, 2008; De Robertis and Sasai, 1996).

3.2. Development of the CNS in vertebrates

The vertebrate CNS is composed of the brain and the spinal cord. It derives from the dorsal ectoderm, where it initally forms the neural plate. It later turn into the neural tube, by a process called neurulation (Gilbert, 2005). The early neural plate becomes subdivided into a complex grid of moleculary distinct domains of progenitors, each of them expressing a unique combination of transcription factors, which will specify the distinct regions of the CNS. The vertebrate CNS is highly regionalized along both its AP and DV axes. This is evidenced by the different structures found in anterior and posterior ends, and along the DV axis of the neural tube (Reviewed in (Lupo et al., 2006). In the study of vertebrate CNS, two structures have been mainly analyzed, the cerebral cortex and the spinal cord.

3.2.1. The vertebrate cerebral cortex.

The cerebral cortex is formed by neurons and glia, and it is organized into distinct layers and columns. These neural cells are generated by neural stem cells called radial glial cells (RGCs), which are located in the ventricular zone (VZ) and subventricular zone (SVZ). RGCs give rise neurons and glia either directly or indirectly via SVZ intermediate progenitor cells (IPCs). Moreover, the radial fibres of the RGCs also serve as a scaffold for the new originated neurons, that migrate from the ventricular zone (the inner layer of the cortex, where the progenitors are) to the cortical surface (Reviewed in (Franco and Muller, 2013; Kriegstein and Alvarez-Buylla, 2009). Neurons are born in a sequential order, forming a six-layered cortex, with neurons generated first occuping deep layers (lower layers V-VI) and, late generated neurons localised in more superficial layers (upper layers I-III). (Fig. I.2 extracted from (Franco and Muller, 2013).

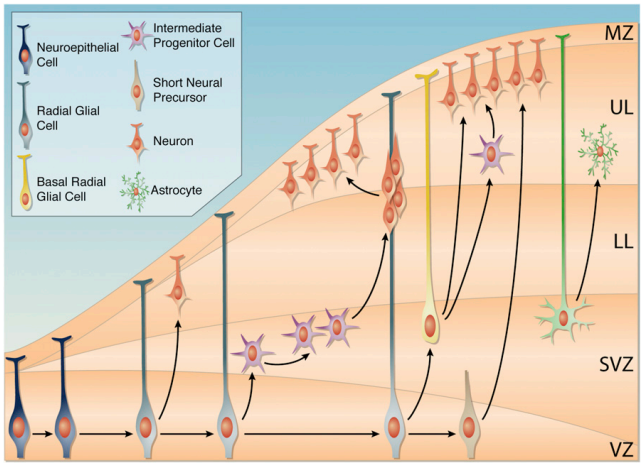


Fig. I.2. Schematic illustration of the vertebral cerebral cortex. In early stages of neocortical development, neuroepithelial cells expand the pools of progenitors before transforming into radial glial cells (RGCs). RGCs typically divide asymmetrically to self-renew and produce either neurons or intermediate progenitor cells (IPCs). IPCs divide symmetrically to generate neurons, or in some cases other IPCs. Short neural precursors are similar to IPCs because they produce neurons, but also similar to RGCs given that they maintain an apical end foot. Basal RGCs (bRGCs) have a basal attachment similar to RGCs, but they have their cell bodies in the SVZ. At the end of neurogenesis, RGCs and bRGCs transform into astrocyte progenitors. (Franco and Muller, 2013). VZ, ventricular zone; SVZ, subventricular zone; LL, lower layers; UL, upper layers; MZ, marginal zone.

It was traditionally assumed that neurons of all layers were produced from a single type of progenitor whose multipotency became restricted over time. That is, at early stages of

development progenitors can generate any class of neurons, but later on, they are only able to give rise to more superficial neurons (Noctor et al., 2001; Rakic, 1988). This would indicate a model in which a common progenitor changes restriction over time. However, several observations has led to propose alternative scenarios in which separate or multiple fate-restricted progenitors might exist for lower and upper layer neurons (Karten, 2013; Kuan et al., 1997).

In fact, the transcription factor Cux2 was proposed as a distinguishing element between two classes of progenitor cells. In this way, Cux2- progenitors generated deep-layer neurons, whereas Cux2+ progenitors primarily produced neurons in the superficial layers (Franco et al., 2012). However, Guo et al., (Guo et al., 2013) have recently reported that the progenies of Cux2+ RGCs contained both lower and upper-layer cortical neurons. This indicated that Cux2+ RGCs were multipotent progenitors and might not be fate-restricted to solely produce upper-layer neurons.

These studies only confirm that there are still many unresolved questions concerning the possible heterogeneity of the progenitor cells of the cortex. (Reviewed in (Han and Sestan, 2013).

3.2.2. The vertebrate spinal cord.

The spinal cord comprises the caudal region of the CNS. As development proceeds patterning along the AP and DV axes commences and cells acquire regional identities determined by their localizations within the neural tube (Reviewed in (Lupo et al., 2006). The regionalization of the DV axis is defined by counteracting gradients of Wnt and BMP signals, that promote dorsal identities; and Shh signaling, that induces ventral identities. (Fig. I.3, extracted from (Ulloa and Briscoe, 2007). The distribution of these long-range signals within the DV axis allows the differential expression of several transcription factors such as *Pax6, Pax7* or *Nkx2.2*, in the progenitors.

This combinatorial expression of the transcription factors establishes spatially limited domains along the DV axis, that each will produce a distinct neuronal subtype. Motoneurons originate at ventral regions, whereas sensory neurons do so at dorsal. Yet, it is still poorly

understood how precise combinations of these transcription factors function in individual cells to give rise to specific cell types.

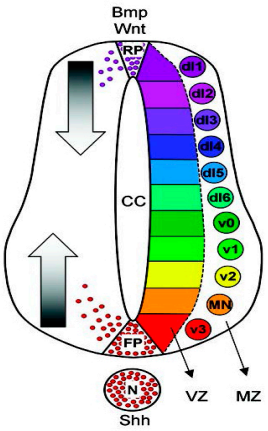


Fig. I.3. Schema of a section of the spinal cord. Proliferative neuroprogenitors are located medially adjacent to the central canal (CC) in a region known as the ventricular zone (VZ). Postmitotic differentiated neurons are located laterally in a region known as the mantle zone (MZ). Distinct neural subtypes are generated from different domains of progenitors arrayed along the DV axis. Individual progenitor domains are identified by the expression of different combinations of transcription factors. The spatially pattern of transcription factor expression in progenitors depends on the action of counteracting gradients of Bmp, Wnt and Shh. N, notochord; RF, roof plate; FP, roof plate. Dorsal is up and ventral is down. (Ulloa and Briscoe, 2007).

3.3. The CNS of *Drosophila.*

Drosophila CNS can be divided into the brain and the subesophageal ganglion, and the ventral nerve cord (VNC), the equivalents of the vertebrate brain and spinal cord, respectively. It originates from a ventrolateral sheet of ectodermal cells named neuroectoderm. As the rest of the fly body, the CNS is segmented, that is, it is formed by repetitive units. The brain consists of 3 brain segments: B1 (protocerebrum), B2 (deutocerebrum) and B3 (tritocerebrum). The subesophageal ganglion is composed of three suboesophageal segments (S1-S3). The VNC is formed by 3 thoracic (T1-T3) and 10 abdominal segments (A1-A10). Each segment is bissected by the midline to form two hemisegments, which are mirror images of each other. The hemisegment is the smallest developmental unit in the VNC, and they are denominated hemineuromeres. The VNC is also commonly denominated ventral ganglion.(Fig. I.4 extracted from (Ito, 1995).

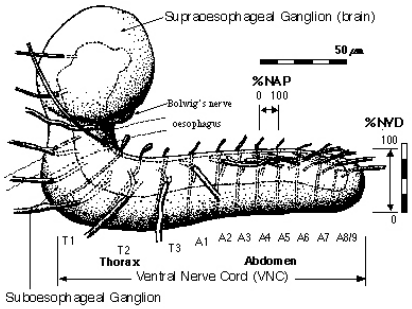


Fig. I.4. Drawing of the central nervous system of a first instar larva of *Drosophila*. (Lateral view). The CNS consists of the brain, subesophageal ganglion and the ventral nerve cord, which contains 3 thoracic and 10 abdominal segments. (Ito, 1995).

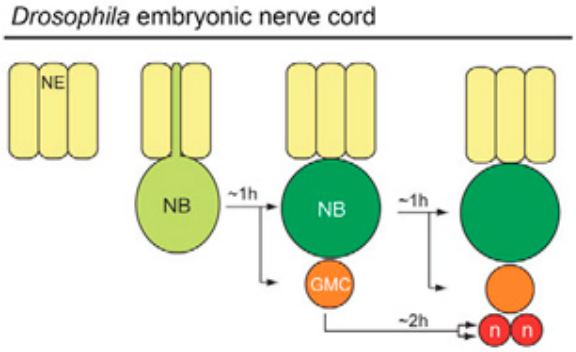


Fig. I.5. *Drosophila* neuroblasts. Neuroectodermal cells (NE) give rise to neuroblasts (NBs) by delamination, and each NB divides in a bud off fashion to generate a ganglion mother cell (GMC). This will typically divide one to generate two postmitotic neurons and/or glia. Approximate cell cycle times are given in hours. (Doe, 2008)

Nervous progenitor cells are called neuroblasts (NBs). They delaminate from the neuroectoderm and divide asymmetrically, self-renewing and budding off a smaller daughter cell called ganglion mother cell (GMC). Due to the asymmetrical partition of NB determinants during division, only the GMC inherits the homeodomain protein Prospero (Pros), which acts as a binary switch to repress cell division and proliferation genes and activate differentiation programs (Choksi et al., 2006). Therefore, the GMC typically divides just once to give rise 2 neurons and/or glial cells. (Fig. I. 5, modified from (Doe, 2008).

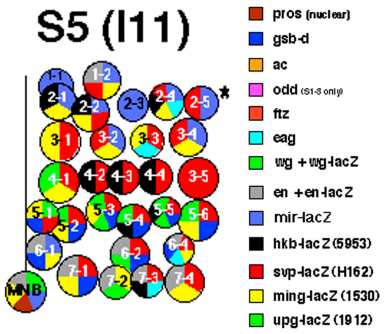


Fig. I.6. Neuroblast map. Schema of an hemisegment (right side) of a late stage 11 embryo, showing the 30 NBs per hemisegment labelled by the different molecular markers. Anterior is up.

During embryonic neurogenesis, patterning genes act in the anteroposterior (segmental genes) and dorsoventral (columnar genes) axes of the embryo to create a cartesian grid of positional information from which NBs delaminate. Each NB acquires a unique identity depending on the position and the time it delaminates into the embryo, and each expresses a unique combination of molecular markers. There are 5 waves of delamination, starting at late embryonic stage 8, that end up in the formation of a map of 30 NBs per hemisegment by late stage 11 (reviewed in (Skeath and Thor, 2003). (Fig. I.6, modified from http://www.neuro.uoregon.edu/doelab/nbmap.html).

Each hemisegment initally contains the same invariant array of 30 NBs, consequently, serially homologous lineages are generated, although some segment-specific differences also exist. In fact, subesophagic and most posterior abdominal segments (A8-9) possess a lower number (Birkholz et al., 2013a). It is now accepted that the expression of homeotic *Hox* genes is behind this regional divergences (Birkholz et al., 2013b). (Reviewed in (Estacio-Gomez and Diaz-Benjumea, 2013; Rogulja-Ortmann and Technau, 2008). Within this 30NBs map, each NB is assigned a number which corresponds to the row and column it occupies, for example, NB5-5 is located in row 5 and column 5.

Lineage-tracing experiments by injecting a fluorescent dye in NBs (DiI labelling), showed that each NB lineage produced a unique and reproducible clone of progeny, and that many NBs are multipotent, being able to generate different types of neurons and glia. Also, the number of cells generated by each lineage is specific, in this way, MP2 (Midline progenitor) represents the smallest lineage with only 2 cells, whereas NB7-1 has the longest lineage and gives rise to around 20 GMCs (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997).

Although nervous progenitor cells are all commonly denominated NBs, these can be subdivided according to the nature of the cell types produced. In this way, there are progenitors that only generate glia, glioblasts. In *Drosophila* these are: NB6-4 Abdominal (A) and LGB (Longitudinal glioblast); progenitors that only generate neurons (neuroblasts) and progenitors that give rise to a mixture of both neurons and glial cells (neuroglioblasts), in *Drosophila* these are: NB1-1A, NB1-3, 2-2 Thoracic (T), NB 5-6, NB6-4T and NB7-4 (Beckervordersandforth et al., 2008).

There are two broad classes of cell types in the nervous system, neurons, which process the information, and glia, which provide neurons with metabolic and mechanical support. There are three types of neurons: sensory neurons, which receive signals from sensory organs; interneurons (INs), which receive and send signals to other neurons; and motoneurons (MNs), which exit the VNC to innervate muscles or release neuropeptides within the hemolymph or glands. Embryonic glia is classified into three groups based on their position and morphology: surface glia, which form a layer around the CNS or peripheral nerves; cortex glia, which ensheath neuronal cell bodies; and neuropil glia (Ito, 1995). The array of 30 NBs per hemisegment will generate during embryonic neurogenesis, around 350

cells, approximately 290 of them will differentiate into interneurons, around 30 will do into motoneurons and around 30 into glial cells.

The terminal identity of a neuron, and ultimately its successful functioning within a neural circuit is defined by a set of characteristic traits that include: the position of the soma, the axonal projection pattern, the dendritic morphology, the expression of ionic channels, the presence of neurotransmitter receptors and the type of neurotransmitter secreted. Many of these features are set up during development, therefore, organisms must coordinate great numbers of regulators in a highly precise spatial and temporal manner. However, there are few cases in which the genetic and molecular mechanisms required to specify a given cell fate are known. (Reviewed in (Jessell and Sanes, 2000; Shirasaki and Pfaff, 2002)).

Besides this positional information provided by the segmental and columnar genes, there is also a temporal axis of information that contributes to increase cell diversity within the NBs. This temporal identity depends on the transiently expression of a series of temporal transcription factors, the so-called temporal genes.

Summing up, each NB will acquire a unique fate depending on its spatial and temporal identity and, cell-autonomously will develop a specific lineage characterized by the number and type of neural cells generated. (Reviewed in (Maurange, 2012; Pearson and Doe, 2003).

3.4. Spatial specification of *Drosophila* CNS: antero-posterior axis.

The main axes of the developing fly are defined before fertilization by a complex exchange of signals between the oocyte and its surrounding follicle cells. Maternal mRNAs are placed in different regions of the embryo, and set up morphogen gradients that establish the anteroposterior and dorsoventral axes of the embryo. Thus, the sequential action, in the AP axis, of the maternal and zygotic genes (gap, pair-rule, segment polarity and *hox* genes) modelate the genetic expression within the neuroectoderm, and allow that NBs situated in different positions acquire distinct cell fates (Alberts, 2002).

It is known that most of gap and pair-rule genes become downregulated within the embryo before neurogenesis, exceptions are *fushi-tarazu* (*ftz), odd-skipped (odd)* and *huckebein* (*hkb)*, which are later expressed in some NBs. Segment-polarity genes are expressed in the neuroectoderm and are maintained in the early-born NBs and their progeny (Skeath, 1999). Mutations in these genes lead to row-specific alterations in the generation and/or specification of the NBs in which they are expressed. For instance, *gooseberry* (*gsb)* is expressed in NBs of row 5 and 6, and, in *gsb* mutants, no row 5 NBs form and in the case they do, they are mispecified towards a row 4 identity (Bhat, 1999; Skeath, 1999).

3.4.1. Homeotic *Hox* genes in *Drosophila* CNS.

Hox genes were first discovered in *Drosophila*, but are present in most animal phyla studied. They are expressed in discrete subdomains from the head-to-tail axis of organisms and confer cellular and tissue identities. Most typically, mutation of a specific Hox gene results in the transformation of part or all of a segment into the likeness of another segment, a phenomenon called homeotic transformation. E.g. mutation of gain-of-funtion of *Antennapedia (Antp)*, a Hox gene that is necessary for the development of an adult leg, leads to the transformation of the antennae into legs (Lewis, 1978).

They encode homeodomain transcription factors and are typically organized in gene complexes. There are two of these complexes in *Drosophila*: the *Antennapedia (ANTP-C)* that specifies segments in the head and anterior thorax, and includes the following genes: *labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr)* and *Antennapedia (Antp*); and *Bx-C* complex, that specifies segments of the posterior thorax and abdomen, and includes the genes *Ultrabithorax (Ubx), abdominal-A (abd-A)* and *Abdominal-B (Abd-B).* Accordingly, there are at least four Hox complexes in vertebrates (Hurley et al., 2005). Besides, in order to increase their target specificity, Hox proteins sometimes make use of cofactors that bind DNA cooperatively with them and help them with the selection of the DNA-binding site. So far, the best-characterized cofactors are all TALE (three amino acid loop extension) homeodomain proteins, in *Drosophila* these are *Extradenticle (Exd)* and *Homothorax (Hth).* (Reviewed in (Mallo and Alonso, 2013; Mann et al., 2009).

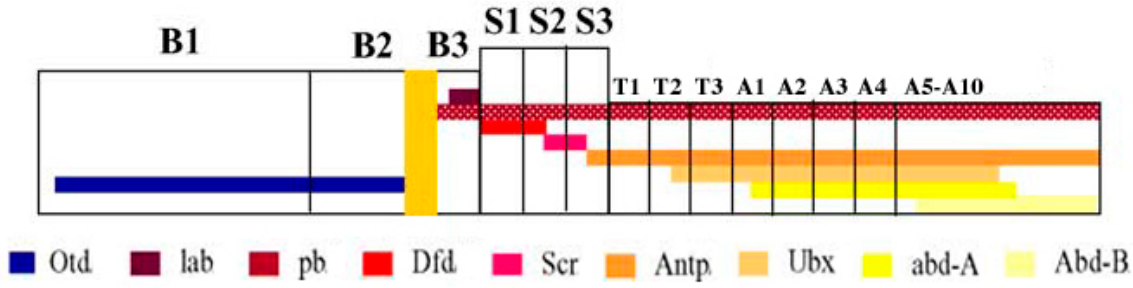


Fig. I.7. *Hox* gene expression patterns in the developing CNS of *Drosophila* The are two complexes of *Hox* genes in *Drosophila*. The Antennapedia complex is formed by *lab, pb, Dfd, Scr* and *Antp*, and specifies segmental indentity in the brain and anterior thorax. The Bithorax complex consists of *Ubx, abd-A* and *Abd-B* and specifies segments of the posterior thorax and abdomen. Expression domains are color coded. Schematic of a stage 14 embryo. Anterior is to the left. Brain:B1-3. Subesophageal ganglion: S1-3. Thorax:T1-3 and Abdominal segments: A1-A10. Modified from (Reichert, 2005).

In *Drosophila*, Hox genes are expressed in the neuroectoderm of the corresponding segments, but as soon as NBs delaminate their expression fades away to be later reactivated in individual cells, NBs or postmitotic neurons, as part of their genetic combinatorial code (Karlsson et al., 2010). This differs from their expression in the ectoderm in early embryonic stages, where once their ON or OFF condition is established, it will be maintained during the rest of development (reviewed in (Gellon and McGinnis, 1998; Lawrence and Morata, 1994). However, this behaviour is similar to the one observed in the vertebrate CNS, where some Hox genes are specifically expressed over a narrow time window during early development (Philippidou and Dasen, 2013).

The expression pattern of *Hox* genes within the CNS appears in the same corresponding segments as in the cuticle (Fig. I.7, extracted from (Reichert, 2005)). Within the VNC, *Ubx* is expressed from the posterior half of T2 to the anterior half of A7 (parasegment (PS) 5 to PS12), *abd-A* is expressed from the posterior half of A1 to the anterior half of A7 (PS7-PS12), and *Abd-B* is expressed from the posterior half of A4 to A9 (PS10-PS14). (Reichert, 2005). Besides their early role in shaping wide cellular territories, *Hox* genes have been shown to actively participate in individual cells controling several processes in the CNS such as: programmed cell death (Bello et al., 2003; Birkholz et al., 2013b; Miguel-Aliaga and Thor, 2004; Rogulja-Ortmann et al., 2008), proliferation (Karlsson et al., 2010; Prokop et al., 1998; Tsuji et al., 2008), and cell specification (Berger et al., 2005; Birkholz et al., 2013b; Estacio-

Gomez et al., 2013; Karlsson et al., 2010; Prokop and Technau, 1994; Rogulja-Ortmann et al., 2008; Suska et al., 2011). During the regulation of the latter, *Hox* genes can act at the level of the neuroectoderm, neuroblasts and/or postmitotic progeny (reviewed in (Estacio-Gomez and Diaz-Benjumea, 2013; Rogulja-Ortmann and Technau, 2008)

3.5. Spatial specification of *Drosophila* CNS: dorso-ventral axis.

The dorsoventral boundaries of the neuroectoderm are defined by the differential activation of the signalling pathways BMP in dorsal regions, and NF-kB (Dorsal) in ventral areas. Another important pathway that act in this process is that of EGF, which, due to the localized expression of *rhomboid* in the ventral and intermediate neuroectoderm, a selective activation of EGF signalling takes places exclusively within this region. This event further subdivides the neuroectoderm into a ventral high Dorsal/EGFR region, an intermediate low Dorsal/EGFR region and a lateral non-signalling area. (Fig. I.8, modified from (von Ohlen and Doe, 2000).

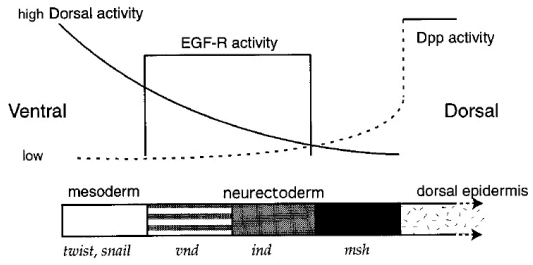


Fig. I.8. Summary of dorsal-ventral patterning in the *Drosophila* embryo. Schematic shows the limits of Dorsal, Egfr, and Dpp signaling. Dorsal is active ventrally in tissues that give rise to mesoderm and ventral ectoderm. Egfr signalling is active in the intermediate and ventral columns (*vnd* and *ind* columns). Dpp signalling is active dorsally, outside the neuroectoderm; dashed line indicates that the activity of Dpp is lower but uncharacterized within the neuroectoderm. (von Ohlen and Doe, 2000).

Each of these regions of the neuroectoderm are defined by the expression of the so-called columnar genes. They are homeobox genes and in order of more ventral towards lateral pattern of expression, they are *vnd*, *ind*, and *msh*. The expression of these genes has been shown to be essential for the specification and/or generation of the NBs and their progeny in which they are expressed. In *vnd* and *ind* mutants around only 10-20% of NBs are formed, and when they do they are misspecified towards an intermediate fate change, in the case of *vnd*

mutants, and towards ventral or dorsal fates, in *ind* mutants. In *msh* mutants, dorsal NBs form, but they are often mispecified, sometimes in a manner that is consistent with a dorsal to intermediate fate change (Isshiki et al., 1997; von Ohlen and Doe, 2000), although *ind* expression does not expand dorsally. Activation and delimitation of *vnd* expression depend on Dorsal, whereas initiation and maintenance of *ind* expression require both Dorsal and EGFR signaling pathways, but not Dpp activity. Additionally, *vnd* represses *ind* in its ventral border. *msh* is expressed in a dorsoventral domain that has low *vnd, ind* and Dpp activity, and overexpression of these genes result in *msh* inhibition. Thus, the borders of *msh* are defined by repression: *vnd* and *ind* ventrally, and Dpp dorsally (von Ohlen and Doe, 2000).

Although the identification of the columnar genes provided a nice model of how the neuroectoderm is patterned along the DV axis, the precise roles of these genes in NB specification is far to be understood.

3.6. Temporal control in the embryonic CNS of *Drosophila.*

Cell type diversity within the lineage of each NB is achieved by a temporal control mechanism. This temporal control is established by the sequential and transiently expression of a series of transcription factors in embryonic NBs, which defines different temporal windows. These are the zinc finger protein Hunchback (Hb), the zinc finger protein Krüppel (Kr), the Pou-domain proteins Pdm1 and 2 (herein named Pdm), the zinc-finger protein Castor (Cas) and the bHLH protein Grainy-head (Grh). (Baumgardt et al., 2009; Isshiki et al., 2001; Kambadur et al., 1998). Each transcription factor is expressed at a different temporal window in the NB during development, and specifies the identity of the cells generated in that window. The genetic profile of expression present in the NB is mostly inherited to its progeny. These factors are expressed in an invariable order in most NBs of the VNC, although some of them do not start the series with a Hb window (Isshiki et al., 2001), and not all terminate with Grh (i. e. NB5-6 A). (Baumgardt et al., 2009).

It is important to note that temporal genes do not drive identity towards a specific cell type. They can equally generate MNs, INs or glial cells (Isshiki et al., 2001).

An important property of the temporal genes is that they cross-regulate each other. Loss- and gain-of-function experiments have revealed that each factor has the ability to activate the next factor in the series, and inhibit either the previous or the next plus one. In that way, Hb represses *pdm* and *cas* transcription, and Kr represses *cas*, Pdm represses *Kr*, Cas represses *pdm*, and Grh represses *cas*. (Baumgardt et al., 2009; Grosskortenhaus et al., 2006; Isshiki et al., 2001; Kambadur et al., 1998; Tran and Doe, 2008). (Fig. I.9C). These intrincated regulations between the different factors provide robustness to the temporal series and ensure that, at a given time, earlier temporal genes are rapidly switched off and avoid precocious expression of later temporal genes of the series. However, this model does not fully explain the phenotypes observed *in vivo* when these genes are eliminated or overexpressed. (Fig. I.9A and B, modified from (Jacob et al., 2008; Maurange, 2012).

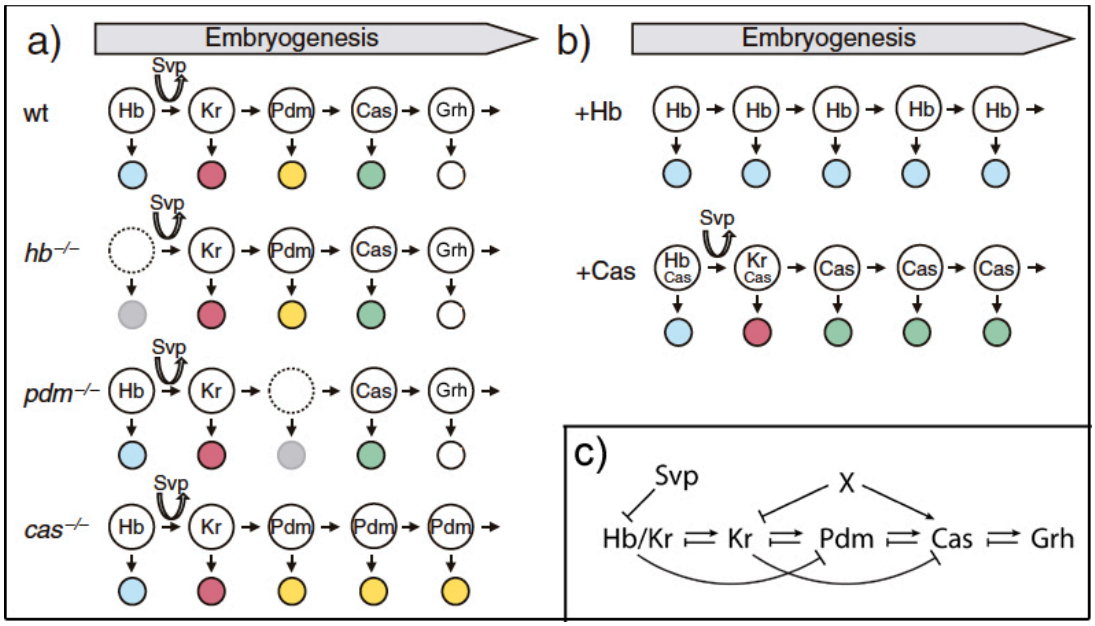


Fig. I.9. Temporal sequence in *Drosophila* NBs. (**A**) Loss-of-function of a single temporal gene leads either to the skipping of the mutated progeny (*hb* and *pdm*) or to stalled temporal series progression (*cas*). (**B**) Continuous misexpression of any of the temporal genes produces supernumerary progeny with the corresponding temporal identity. (**C**) Summary of the known regulatory relationships between the identified temporal genes. The existence of an hypothetic x factor has been proposed to explain the phenotypes seen *in vivo*. (Jacob et al., 2008; Nakajima et al., 2010).

When *hb, Kr, pdm* and *grh* are eliminated, only the neurons generated under the mutated temporal factor disappear, without altering the previous or posterior identities. For example, in *pdm* mutants, Hb and Kr positive neurons are produced, as well as the Cas positive neurons.

An exception to this would be *cas* mutation, in this case nor the Cas positive neurons are formed nor the Grh positive ones. These results indicate that loss of *hb, Kr* and *pdm* only has a minor effect on the progression of the series, and bring up the following questions: how is *Kr* activated in *hb* mutants? or *pdm* in *Kr* mutants? Or *cas* in *pdm* mutants? Therefore, it has been proposed that an upstream timer might be superimposed. In fact, a recent study using a Boolean-type computational model, suggests the existence of an hypothetic x factor that would explain why progression of the series is not affected by the loss of *hb, Kr*, or *pdm* (Nakajima et al., 2010). This x factor would activate *Kr* and repress *cas*, whereas *pdm* and *cas* would be transcribed by default (Fig. I.9C). Conversely, continuous misexpression of a temporal gene prevents progression through the series by blocking expression of late temporal factors. (Fig. I.9B).

Some insight about the temporal series was gained when it was discovered how the Hb window became downregulated. The COUP-family protein Seven-up (Svp), which is an orphan receptor, acts as a *hb* transcriptional repressor (Kanai et al., 2005). *svp* mRNA is actively transcribed from early on in the NB, but it is mostly maintained within the nucleus, which prevents its translation. After NB division, *svp* mRNA diffuses into the cytoplasm, becomes translated and downregulates *hb* activity, which leads to progression to the Kr window (Mettler et al., 2006). Recently, two additional DNA-binding proteins of the Pipsqueak family, Distal antenna (Dan) and Distal antenna-related (Danr) have been shown to act in parallel to Svp in the process of Hb downregulation (Kohwi et al., 2011). In this scenario, both Svp and Dan/Danr are classified as switching factors, in terms that they facilitate the progression within the different temporal windows. Besides, other subtemporal factors have also been proposed. In NB5-6T, where there is a long Cas window, it has been shown that Nab/Sqz (Baumgardt et al., 2009), and more recently, also Svp (Benito-Sipos et al., 2011), act to subdivide it in order to generate diffferent cell types.

3.7 Larval neurogenesis of *Drosophila* CNS.

As *Drosophila* is an holometabolous insect, that is, an insect that has a larval and a pupal stage prior to the adult stage; there are two periods of neurogenesis that account for the different nervous systems needed in larval and adult stages. In this way, the embryonic neurogenesis

gives rise to the larval nervous system, and the postembryonic or larval neurogenesis generates most of the adult nervous system (10% of the adult CNS neurons corresponds to remodelled larval cells). These two phases of neurogenesis are separated by a non-proliferative state, called quiescence. (Reviewed in (Maurange and Gould, 2005)). (Fig. I.10). Most NBs stop proliferation by the end of embryogenesis, and depending on the type of NBs, re-enter neurogenesis at different larval stages. For example, brain NBs do so at mid first instar larvae, thoracic NBs at the beginning of second instar larvae, and abdominal NBs at the beginning of third instar larvae. Mushroom bodies NBs represent an exception because they do not stop dividing during larval stages.

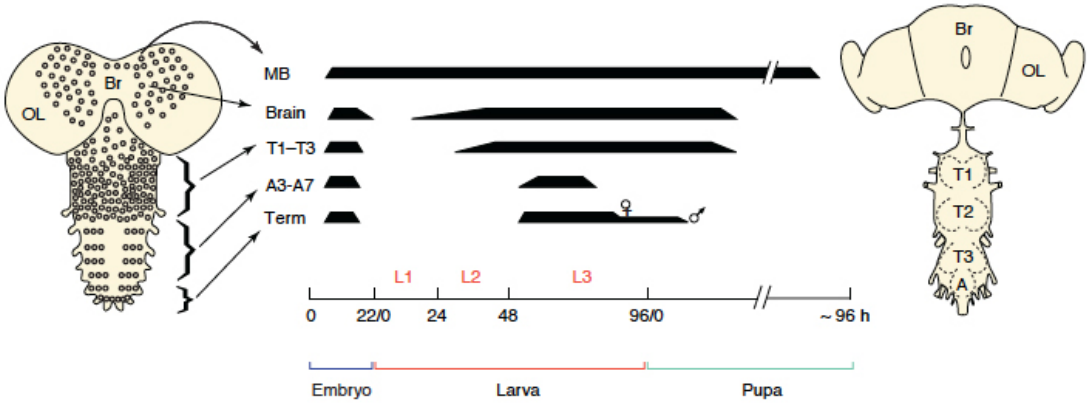


Fig. I.10. Patterning of neuroblast activity along the anteroposterior axis. Representation of proliferation times (S-phase by incorporation of Bromodeoxyuridine, black bars) of NBs from various positions, throughout development. MB NBs never stop dividing. The rest of NBs stop proliferation by the end of the embryonic phase, enter quiescence and resume proliferation between first and second instar larvae. Cartoons depicting the shape of a larval CNS (left) and an adult CNS (right). Abbreviations: A, abdominal segments; Br, brain, MB, mushroom body; OL, optic lobe; T, thoracic segments; Term, terminal segments. Larval development is divided into three instars:L1, L2 and L3. (Maurange and Gould, 2005).

Additionally, not all NBs undergo this second round of neurogenesis; nearly all brain and thoracic NBs do, but in central abdominal segments (A3-A7), most NBs die by apoptosis at the end of the embryonic neurogenesis, and only 3 remain per hemisegment and undergo quiescence. This segment-specific elimination pattern depends on an input from Abd-A (Prokop et al., 1998). These postembryonic NBs (pNBs) can be easily identified by their positions in the VNC, and they are named accordingly, ventromedial (vm), ventrolateral (vl) and dorsolateral (dl). (Truman and Bate, 1988). However, their identities have not been addressed to their corresponding embryonic NBs (eNBs). It has been found that temporal

transitions are suspended in quiescent NBs, and this is independant of the temporal factor expressed (Tsuji et al., 2008). Reentry into the cell cycle depends in the availability of sufficient nutrients in the food, as sensed by the fat body and transmitted to the NB by glial insulin (Chell and Brand, 2010; Sousa-Nunes et al., 2011). During the metamorphic pupal stage, all NBs terminate division. Therefore, no neural proliferaton is observed in the adult animal (Reviewed in (Maurange and Gould, 2005). Although it has been recently reported the existence of quiescent NBs in the adult brain (Fernandez-Hernandez et al., 2013).

It has been shown that pNBs redeploys some of the embryonic temporal factors, namely *cas, svp* and *grh*, which enable NBs to terminate neurogenesis at a proper time: via cell cycle exit, used by brain and thoracic pNBs, or apoptosis, employed by abdominal pNBs. In both cases, the transient expression of temporal factors in the pNBs installs the competence to timely terminate NB divisions, and consequently, manipulated NBs blocked in a young temporal identity never stop dividing. In brain and thoracic pNBs the delocalization of Pros from the membrane to the nucleus produces exit from the cell cycle, and in abdominal pNBs a pulse of Abd-A mediates apoptosis (Maurange et al., 2008).

3.8. Neural specification in *Drosophila*: Abdominal Leucokinergic neurons (ABLKs).

A fundamental question in developmental neurobiology is to dissect the molecular and genetic mechanisms that govern neural specification, there are a very few cases in which the NB progenitor of a neuron and the genetic mechanisms underlying its specification are known. In addition, the lack of availability of terminal molecular markers with restricted expression makes it difficult to unequivocally identify specific subsets of neurons.

The *Drosophila* embryonic/larval VNC contains around 10,000 cells (Schmid et al., 1999). Of these, around 150 are peptidergic, as defined by the expression of one of 30 identified neuropeptides (Park et al., 2008). Because of their highly restricted expression, neuropeptides have emerged particularly useful markers for addressing the mechanisms controlling neuronal subtype specification in both vertebrates and invertebrates (Brohl et al., 2008; Thor, 2008). In particular, studies of one subgroup of peptidergic neurons, the thoracic Apterous neurons, have resulted in a detailed understanding of how spatial and temporal cues are

integrated into combinatorial regulatory codes of cell fate specification (Allan et al., 2005; Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Miguel-Aliaga et al., 2004). To shed light on whether such genetic cascades are commonly used during neuronal subtype specification, we have initiated studies of another peptidergic cell type: the *Drosophila* abdominal leucokinergic neurons (ABLKs).

3.9. Leucokinergic system.

The description of leucokinergic neurons in *Drosophila melanogaster* was first performed by Cantera and Nässel (Cantera and Nassel, 1992). They raised an antiboby against Leucokinin I (Lk I) (Nassel and Lundquist, 1991), and studied peptidergic neurons and their innervation patterns in larvae and adults of three species of higher dipteran insects, including *Drosophila*.

Between this and other works (Herrero et al., 2007; Herrero et al., 2003), it was proposed that the larval leucokinergic system at third instar larva is formed by four different sets of neurons:

- one pair of prominent Lk neurons in the brain lobes, termed LHLK to reflect their location in the lateral horn in adults.
- a cluster of 2-4 weakly stained Lk neurons that was often observed in the anterior brain lobe, named ALKs. These started to fade by late third instar, and were absent in adults.
- two pairs of Lk neurons located in the third subesophageal neuromere, called SELKs.
- seven pairs of neurons in the most anterior abdominal neuromeres of the ventral ganglion, hereafter called ABLKs.

In adults, they found one pair of LHLKs and one pair of SELKs. Besides, they also described an increase in the number of ABLKs cells, eight to ten pairs of Lk-immunoreactive cells in the abdominal neuromeres (Herrero et al., 2003). ABLKs extend axonal projections both within the CNS and to the periphery. The axon projection to the periphery terminates on lateral muscle 8. The terminal of ABLKs is distinct from those of MN since it contains no bouton-like structures and is classified as a type-3u terminal, that is, it lacks synapsin and subsynaptic reticulum (Landgraf et al., 2003).

3.10. Functions of leucokinergic neurons.

Leucokinins form a neuropeptide family of amidated peptides found in most invertebrate species (Nassel, 2002). They were initially identified as neurohormones that increase hindgut motility in some insects and Malpighian tubule fluid secretion in all insects species studied so far (Chen et al., 1994; Coast et al., 1993; Hayes et al., 1989; Hewes and Taghert, 2001; Holman and Cook, 1983; Schoofs et al., 1992; Terhzaz et al., 1999). Besides these functions, the presence of projections of abdominal leucokinergic cells in the heart, abdominal spiracles and neurohemal regions of the abdominal wall, led authors to suggest additional roles for Lk in cardioregulation and respiration (Cantera and Nassel, 1992). Lk receptors have been found in the brain, ventral nerve cord, renal tubules and digestive tract (Radford et al., 2002; Veenstra et al., 2008). The neural functions of Lk are beginning to be uncovered.

It has been shown that Lk is implicated in the regulation of meal size. Adult flies with mutations in Lk or its receptor, increased their meal size while reducing the frequency of food intake. Thereby the total amount of calories in mutant flies was similar to the one in *wild-type*. This phenotype was likely due to the impaired communication of gut distensions signals to the brain (Al-Anzi et al., 2010).

In another work, Lopez-Arias et al., (Lopez-Arias et al., 2011) showed that electrically silencing Lk neurons altered both olfactory and gustatory responses, and proposed that Lk modulated chemosensory responses through leucokinergic brain neurons.

A recent study has confirmed the role of Lk in the regulation of water homeostasis in vivo (Cognigni et al., 2011). In this paper the authors developped a quantitative method based on defecation behaviour to study the role of *Drosophila* intestine in the regulation of nutrient intake, fluid, and ion balance. Overactivation of Lk neurons resulted in increased diuresis, as shown by the presence of lighter (less concentrated) and more abundant deposits. Conversely, genetic silencing of Lk neurons resulted in concentrated deposits of much smaller size. What is more, a selective downregulation of Lk expression and its receptor ubiquitously or specifically in the nervous system confirmed that Lk acted as a central neurohormone on its receptor in non-neural tissues to control fluid balance, in a manner analogous to mammalian vasopressin (Nussey and Whitehead, 2001).

Moreover, Luo et al., (Luo et al., 2013) have found that ABLKs are one of the neuroendocrine, Dimmed (Dimm) positive cells that, through the insulin/insulin-like growth factor signaling pathway, can undergo size variations that provide plastic scaling and protection of secretory activity during development or in the adult life; this would ensure an appropiate hormone production for the body volume. The gene dimm encodes a basic helix-loop-helix (bHLH) protein required for neuroendocrine cell differentiation (Allan et al., 2005; Hamanaka et al., 2010; Hewes et al., 2003).

Lastly, it has been shown that ABLKs express one of the five receptors for the neurotransmitter serotonin (5-HT), which led authors to propose ABLKs as downstream targets of 5-HT involved in turning. 5HT modulates larval turning by regulating the activity level of downstream ABLK neurons and secretion of the neuropeptide Lk (Okusawa et al., 2014).

4. Objectives

The aim of this thesis was the analysis of the mechanisms of specification of ABLKs. We wanted to unravel the genetic and molecular mechanisms behind this process. To that end, we set the following goals:

1. Analysis of the expression pattern of ABLKs.
2. Identification of the progenitor neuroblast of the ABLKs.
3. Identification of the temporal factors involved in ABLKs specification.
4. Study of the role of programmed cell death and Notch signaling pathway in the specification of ABLKs.
5. Screening for genes involved in ABLKs specification.
6. Analysis of the expression pattern of ABLKs from third instar larva to adult stage.
7. Determination of the origin of the ABLKs present in the adult CNS.
8. Analysis of the role of *Hox* genes in the segment-specific appearance of ABLKs throughout development.

5. Materials and Methods

5.1.Fly stocks.

Fly stocks were cultured under standard conditions (23-25ºC and 60% of relative humidity). The following fly strains were used in this work. Most genetic elements are described in Flybase (Drysdale et al., 2005) or in the appropriate references provided below. Whenever feasible null or strong hypomorph alleles were chosen. Unless otherwise stated, flies were obtained from the Bloomington *Drosophila* Stock Center.

ab^{1D}, *abd-A*^{M1} (Sanchez-Herrero et al., 1985), *Abd-B*^{M1} and *Abd-B*^{M5} (obtained from E. Sánchez-Herrero, CBM, Madrid, Spain), *Act5C>stop>*β*-galactosidase, Antp*^{14} (Lewis et al., 1980) and *Antp*^{25} (provided by D. Karlsson, Linköping University, Linköping, Sweden), *ap*^{P44} (gift from S.Thor, Linköping University, Linköping, Sweden), *apt*^{K15608}, *asp*^{1}, *Awh*$^{63Ea\text{-}G14}$, *bowl*^{1}, *caps*$^{65.2}$, *cod*^{2}, *cas*^{D1} *and cas*^{D4} (Mellerick et al., 1992) (provided by M. Baumgardt, Linköping University, Linköping, Sweden), *Chip*$^{e5.5}$ and *Chip*$^{g96.1}$ (Pueyo and Couso, 2004) (provided by J.P. Couso, University of Sussex, Brighton, UK), *col*^{1} (obtained from S. Thor), *crol*^{04418}, *da*^{1}, *dac*^{4} (Mardon et al., 1994), *col*^{1} (obtained from S. Thor), *Dfd*^{1}, *dimm*^{Rev8} (Hewes et al., 2000) (obtained from R.S. Hewes, University of Oklahoma, Norman, USA), *dsf*^{f00109}, *EcR*^{Q50st}, *EcR*^{k06210}, *EcR*^{31}, *EcR*^{112}, *elB*$^{3.3.1}$ *noc*^{D64}, *ems*^{1}, *esg*$^{35Ce\text{-}1}$, *esg*^{G66} (Whiteley et al., 1992) (obtained from Y.Hayashi, Tohoku University, Sendai, Japan), *esn*^{f00447}, *exex*^{KK30}, *ey*^{2}, *eya*$^{cli\text{-}IID}$, *eyg*^{SA2} (#558) (gift from N. Azpiazu, CBM, Madrid, Spain), *fkh*^{1}, *ftz*^{5}, *ftz*^{13}, *grn*^{7L}, *grh*^{IM}, *grh*^{370} (Bray and Kafatos, 1991) (provided by A.P.Gould, National Institute for Medical Research, London, UK), *gt*^{E6}, *Df(3L)H99, hb*^{P1} *hb*^{FB} (Isshiki et al., 2001) (gift from C.Q.Doe, University of Oregon, Eugene, USA). *hb*^{FB} is a null allele, whereas *hb*^{P1} is a transgene that is expressed in the anterior Hb gap domain but not in neuroblasts or their progeny, thus this genetic combination removes Hb CNS expression. *hkb*^{2}, *hkb*^{A321R1}, *Hsf*^{1}, *hth*^{5E04}, *Df(3R)Exel6158 (referred to as hth*^{6158}*), htl*^{AB42}, *jing*^{01094}, *jing*^{22F3} (Culi et al., 2006) (provided by J.Culí, CABD, Seville, Spain), jumu^{06439}, *ken*^{02970},*klu*^{212IR51C} (Yang et al., 1997) (provided by W.Chia, Temasek Life Sciences Laboratory, National University of Singapore, Singapore), *kni*^{6}, *kni*^{9}, *Kr*^{1} *Kr*^{CD} (to remove Kr CNS expression: *Kr*^{1} is a null allele, whereas the transgene *Kr*^{CD} is expressed in the central Kr gap domain and weakly in the neuroectoderm, but not in neuroblasts or their progeny) (Isshiki et al., 2001)(gift from C.Q.Doe), *lab*^{4}, *mam*^{GA345}, *mld*^{92} and *mld*^{47} (Neubueser et al., 2005) (obtained from S.Cohen, Institute of Molecular and Cell Biology, Singapore), *nab*^{R52} (Terriente Felix et al., 2007), *Nkx6*^{D25} (Broihier et al., 2004)(provided by R. Urbach, Johannes Gutenberg University, Mainz,

Germany), *numb*^{1}, *odd*^{01863}, *osa*^{2}, *pb*^{10}, *Df(2L)ED773* (this deficiency removes *pdm1* and *pdm2*), *pnr*^{v1}, *pnr*^{vx1} (Ramain et al., 1993)(provided by M. Baumgardt), *pnr*^{vx6}, *prd*^{8}, *Df(3R)rn*^{20} (St Pierre et al., 2002), *rpr*^{XR38} (White et al., 1996)(obtained from K. White, Harvard Medical School, Charlestown, USA), *rtn*^{1}, *Scr*^{4}, *slp1*^{1}, *slp1*^{2}, *spdo*^{G104}, *Df(3R)Dl-KX23* (referred to as *sqz*^{Df}), *sqz*^{ie}, *sqz*^{Gal4} (Allan et al., 2003), *stc*^{05441}, *svp*^{1} (provided by A.P.Gould), *tll*^{1}, *tll*^{l49}, *Df(2R) tsh*^{8}, *toy*^{hdl}, *tup*^{1}, *Df(3R)Ubx*^{109} (Lewis, 1978), *Ubx*^{MX6} (Casanova et al., 1987) and *Ubx*$^{6.28}$ (Beachy et al., 1985)(provided by E. Sánchez-Herrero), *vn*^{10567}, *zfh1*^{00865}, *Df(2R)Exel 7135, Df(3L)BSC558, Df(2R)Exel 6283, Df(3R) BSC499, Df(3R)Exel8154, Df(3R)Exel7309, Df(3R)BSC493, Df(3R)BSC679, Df(3R)BSC509, Df(3R)Exel6179, Df(1)ED7294, Df(2R)BSC350, Df(2R)BSC303, Df(2R)Exel6071, Df(2R)Exel 7166, Df(2R)Exel 6055, Df(2R)BSC266.*

Zinn deficiencies kit: *Df(2L)net-PMF, Df(2L)BSC106, Df(2L)BSC4, Df(2L)BSC16, Df(2L)BSC37, Df(2L)dpp[d14], Df(2L)JS17, Df(2L)BSC28, Df(2L)BSC31, Df(2L)ED250, Df(2L)BSC109, Df(2L)Exel6011, Df(2L)BSC169, Df(2L)Exel6013, Df(2L)ED347, Df(2L)Exel6015, Df(2L)Exel9038, Df(2L)Dwee1-W05, Df(2L)Exel7029, Df(2L)Exel7031, Df(2L)ED499, Df(2L)Trf-C6R31, Df(2L)BSC111, Df(2L)Exel7038, Df(2L)BSC215, Df(2L)BSC143, Df(2L)Exel7048, Df(2L)ED746, Df(2L)BSC36, Df(2L)FCK-20, Df(2L)TE35BC-24, Df(2L)Exel6038, Df(2L)Exel7066, Df(2L)Exel8036, Df(2L)BSC148, Df(2L)BSC256, Df(2L)Exel6045, Df(2L)ED1317, Df(2L)Exel7080, Df(2L)Exel6047, Df(2L)Exel6048, Df(2L)ED1466, Df(2L)Exel6049, Df(2L)BSC151, Df(2L)C', Df(2R)Nipped-D, Df(2R)Exel7092, Df(2R)w45-30n, Df(2R)BSC132, Df(2R)BSC303, Df(2R)BSC281, Df(2R)Exel7124, Df(2R)Exel7131, Df(2R)Exel7135, Df(2R)Exel6063, Df(2R)Exel7142, Df(2R)BSC161, Df(2R)BSC355, Df(2R)Exel7150, Df(2R)ED3923, Df(2R)Exel6082, Df(2R)Px2* (Wright et al., 2010).

lacZ lines: *chinmo*^{k13009}*-lacZ*, *grh*^{06850}*-lacZ*, *gsb-d*^{01155}*-lacZ* (obtained from S.Thor), *hkb*^{5953}*-lacZ, klu*^{09036}*-lacZ, lbe(K)-lacZ* (this transgenic line contains a 2kb genomic fragment of the regulatory region of the *lbe* driving *lacZ*, and reproduces the pattern of expression of *lbe* (De Graeve et al., 2004) (provided by M. Baumgardt), *mirror-lacZ* (obtained from S.Campuzano, CBM, Madrid, Spain), *nab*$^{SH143-}$*lacZ* (Terriente Felix et al., 2007), *sqz*^{02102}*-lacZ, svp*^{H162}*-lacZ, unpg*^{1912}*-lacZ* (Doe, 1992)(provided by R.Urbach) and *wg-lacZ.*

Gal-4/Gal-80 lines: *btd-Gal4* (Estella et al., 2003)(obtained from D.Foronda, Institute of Molecular and Cell Biology, Singapore), *eg*^{MZ360}*-Gal4* (obtained from B. Condron, University of

Virginia, Charlottesville, USA) *elav^{C155}-Gal4* on chromosomes I and III, *en-Gal4, ins^{MZ1407}-Gal4, leucokinin ^{M7}-Gal4* (de Haro et al., 2010)(provided by P.Herrero), *tub-Gal80^{ts}, wg^{MD758}-Gal4* (gift from M. Calleja, Centro de Biología Molecular Severo Ochoa, CBM, Madrid, Spain), *wor-Gal4* (Lee et al., 2006)(obtained from C.Q.Doe), *zfh2^{LP30}-Gal4* (gift from I. Rodríguez).

UAS lines: *UAS-abd-A$^{20\text{-}10\text{-}1}$* (Michelson, 1994)(provided by E. Sánchez-Herrero), *UAS-abd-A-RNAi^{51900}* (Vienna Drosophila RNAi Center, VDRC), *UAS-Abd-B^{m2SG19}* (Castelli-Gair et al., 1994) (provided by E. Sánchez-Herrero), *UAS-Abd-B-RNAi^{12024}* (VDRC), *UAS-Antp* (Hirth et al., 2001)(provided by E. Sánchez-Herrero), *UAS-Dfd^{W4}*, *UAS-dicer* on chromosomes II and III, *UAS-dimm* (Hewes et al., 2003), *UAS-cas* on chromosomes II and III (Kambadur et al., 1998)(obtained from M. Baumgardt), *UAS-flp^{122}, UAS-grh^{15M}* (Baumgardt et al., 2009) (obtained from M. Baumgardt), *UAS-hb^{F4A}, UAS-hb-RNAi^{44892}* (VDRC), *UAS-hth*, *UAS-hth-RNAi* (long form), *UAS-klu, UAS-kni* (Lunde et al., 2003) (provided by E.Bier, University of California, San Diego, USA), *UAS-Kr, UAS-lab, UAS-mad-RNAi^{1013}* (Transgenic RNAi Project), *UAS-nab* on chromosomes II and III (Terriente Felix et al., 2007), *UAS-Nintra, UAS-pb, UAS-pdm* (Grosskortenhaus et al., 2006) (provided by C.Q.Doe), *UAS-p35*, *UAS-Scr, UAS-sqz* (Allan et al., 2003)(obtained from S. Thor), *UAS-svp1$^{1.12}$* (Kramer et al., 1995) (obtained from Y. Hiromi, National Institute of Genetics, Shizuoka, Japan), *UAS-tsh* (provided by H. Skaer, Cornell University, Ithaca, USA), *UAS-Ubx^{IAI}* (Castelli-Gair et al., 1994) (provided by E. Sánchez-Herrero), *UAS-dsRNA-Ubx* (Monier et al., 2005) (provided by D.L.Garaulet, Memorial Sloan Kettering Cancer Center, New York City, USA), *UAS-zfh2-RNAi* (Terriente et al., 2008).

Mutants were kept over *FM7 Act-GFP; CyO, Act-GFP; CyO, Dfd-EYFP; CyO, twi-Gal4 UAS-GFP; TM3, Ser Act-GFP; TM3, Sb Ser twi-Gal4 UAS-GFP*; or *TM6, Sb Tb Dfd-EYFP* balancer chromosomes. Mutants were identified by the absence of GFP expression or, where relevant, by other markers. As *wild-type*, Canton S was often used.

5.2.Gal4/UAS/Gal80 system.

For ectopic expression of genes we used the Gal4/UAS system (Brand and Perrimon, 1993). This system allows expressing a gene at an abnormal time and/or outside the cells where it is normally expressed. Gal4 is a yeast transcriptional activator that selectively binds to the

upstream activation sequence (UAS), and activates transcription of any gene located under its control. Therefore, this technique requires flies containing two constructs. The first is an enhancer trap that expresses Gal4 protein dictated by nearby enhancers. The second construct is a UAS transgenic line that carries the cDNA of the gene of interest that wants to be ectopically expressed. None of these transgenic lines have an effect on their own in *Drosophila*, UAS-cDNA transgenes are not transcribed in the absence of the Gal4 protein. However, when a Gal4 enhancer trap line is crossed to a UAS-cDNA line, the cDNA is expressed in the progeny, specifically in those cells making the Gal4 protein. (Fig. M.1, modified from (St Johnston, 2002)). Gal4/UAS can also be used to knock down genes or label patterns of expression, if UAS sequences contain RNAi of interest genes or reporter genes, respectively.

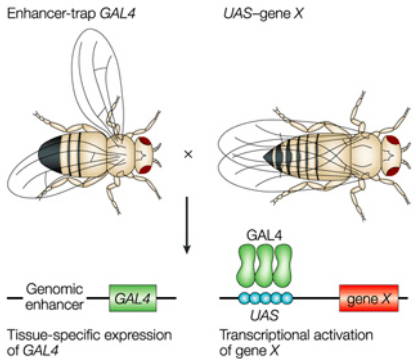


Fig. M.1.The Gal4/UAS system for directed gene expression. The transcriptional activator Gal4 can be used to drive gene expression in *Drosophila* by inserting the upstream activating sequence (UAS) to which it binds next to a gene of interest. In this way, genes can be overexpressed at controlled spatial and temporal patterns (St Johnston, 2002). .

On top of this, the protein Gal80 can regulate the activation of the Gal4/UAS system throughout time. Gal80 also comes from yeast. It binds to the activating region of Gal4 and avoids it to activate transcription of the UAS-cDNA line. There are alleles of Gal80, which are temperature-sensitive, meaning that they are not functional at 29ºC, and therefore Gal4 protein is able to activate transcription of UAS-cDNA. On the other hand, at 17ºC Gal80 is functional and Gal4/UAS is inactivated. (McGuire et al., 2003). This will be extremely useful to ectopically express certain genes only at specific developmental times.

5.3. Flip-out clones.

In order to perform lineage-tracing experiments, we did flip-out clones. Flip-out consists of the excision of a DNA sequence by FLP-induced mitotic recombination between FRT sites that are on the same chromosome flanking the sequence (Struhl and Basler, 1993). FLP (Flippase)

is an Integrase family site-specific recombinase that induces recombination at defined sequences known as FRT sites (Flippase Recognition Target). We have used a line in which the ubiquitous *Actin5C* promoter was separated from the *lac-Z* reporter gene by a flip-out cassette. This cassette consists of CD2, a spacer gene containing a transcriptional stop site, flanked by two FRT sites. Expression of the FLP (UAS-FLP) is under the control of a Gal4 line present in the cells that we want to label. Thus, only these cells will express the FLP, and consequently the *lac-Z* reporter. (Fig. M.2, modified from (Evans et al., 2009)).

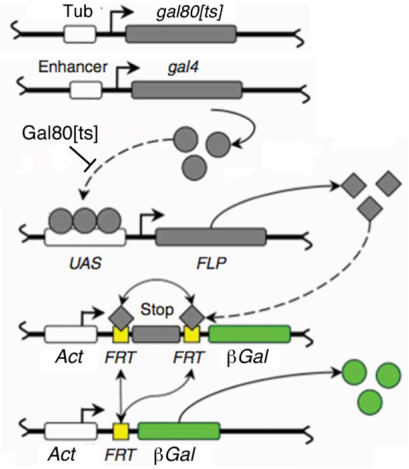


Fig. M.2. Schematic of the lineage tracing experiments performed. Gal4 activates expression of FLP recombinase. Cells expressing FLP recombinase then excise the *FRT*-flanked stop cassette separating the *Act5C* promoter and *β-galactosidase* open reading frame. This initiates the expression of β-galactosidase, which is inherited to all daughter cells. At 18ºC, tubulin-G80 blocks Gal4 mediated transcription. Upon a shift to 29ºC, Gal80 repression is released and Gal4 transcription proceeds. Modified from (Evans et al., 2009).

5.4. Lineage tracing experiments.

Experiment 1: Labelling of *wg-Gal4* expressing cells during 7-9 hours of embryonic development (stages late 11 and 13).

Embryos of genotype *yw UAS-flp^{122}; Act5C>y^{+}>β-galactosidase/wg-Gal4; tub-Gal80^{ts}/+* were collected for 2 hours at 25ºC and shifted to 17ºC. At 7 hours of embryonic development (approximately the end of stage 11), they were moved to 30ºC for 2 hours, and then shifted back to 17ºC. Then as first instar larvae, dissected and stained with anti-LK and anti-β-galactosidase. Only cells expressing *wg-Gal4* between 7 and 9 hours of embryonic development activate flp-mediated recombination of the *Act5C>y^{+}>β-galactosidase* cassette and they and all their progeny permanently express β-galactosidase (*Act5C> β-galactosidase*).

Experiment 2: Labelling of postembryonic NBs and their progeny.

Embryos of genotype *wor-Gal4/Act5C>stop>β-galactosidase; UAS-flp^{122}/tub-Gal80^{ts}* were collected for 2 hours at 17ºC, allowed to grow to early second instar larva and then shifted to 29ºC. They were dissected and stained either as late third instar larva or as adults. Temperature shift at early second instar larva leads to the recombination and constitutive expression of β-galactosidase in pNBs and their progeny, while neurons generated at earlier stages remain unlabelled.

Experiment 3: Labelling of *wg-Gal4* expressing cells during 7-9 hours of embryonic development (stages late 11 and 13).

Embryos of the genotype *wg-Gal4/Act5C>stop> β-galactosidase; UAS-flp^{122}/tub-Gal80^{ts}* were collected over 2 hours at 29ºC, allowed to grow for 7 hours, shifted to 17ºC, and dissected and stained as second instar larvae. In this experiment, only NBs from row 5 are labelled. Given the low efficiency of the Flippase recombinase in recombination of the cassette, very few clones per ganglion were labelled.

5.5. Knock-down and ectopic expression experiments.

Experiment 4: *Ubx, abd-A* and *Ubx abd-A* knock down from first instar larva onwards.

To knock down *Ubx* and *abd-A* in the larval CNS, *elav^{C155}-Gal4; tub-Gal80^{ts}/TM3, Act>GFP* females were crossed with either *UAS-abd-A-RNAi^{51900}; UAS-dsRNA-Ubx Df(3R) Ubx^{109}/TM6B* (provided by D.L.Garaulet, Memorial Sloan Kettering Cancer Center, New York City, USA), *UAS-abd-A-RNAi^{51900}* or *UAS-dsRNA-Ubx Df(3R) Ubx^{109}/TM6B* males. Embryos from the crosses were collected over 24 hours at 17ºC, kept for a further 20 hours at 17ºC, then shifted to 29ºC as first instar larva. Individuals of genotype *elav^{C155}-Gal4/+; UAS-abd-A-RNAi^{51900}*/+; *tub-Gal80^{ts}/ UAS-dsRNA-Ubx Df(3R) Ubx^{109}*, *elav^{C155}-Gal4/+; UAS-abd-A-RNAi^{51900}*/+; *tub-Gal80^{ts}*/+ and *elav^{C155}-Gal4/+; tub-Gal80^{ts}/UAS-dsRNA-Ubx Df(3R) Ubx^{109}* were dissected and stained as mature third instar larvae or adults. Heterozygosis for *Df(3R)Ubx^{109}*, which removes *Ubx* and *abd-A*, increases the efficiency of the knock down. We tested RNAi efficiency by labelling Abd-A anb Ubx. Although Abd-A was completely lost, we detected a low level of Ubx, even in *Df(3R)Ubx^{109}/+;* hence, the result of this experiment has to be interpreted with caution. In fact,

we expected *Ubx* knock down to remove LK from segment A1, but did not observe any phenotype, probably owing to the high level of *Ubx* expression in this segment.

Experiment 5: Misexpression of *abd-A* from first instar larva onwards.
To misexpress *abd-A* from first instar larva, *elav^{C155}-Gal4; tub-Gal80^{ts}/TM3, Act>GFP* females were crossed with *UAS-abd-A$^{20-10-1}$* males. Embryos from the cross were collected over 24 hours at 17ºC, kept for a further 20 hours at 17ºC, then shifted to 29ºC as first instar larva. Individuals of genotype *elav^{C155}-Gal4/+; tub-Gal80^{ts}/UAS-abd-A$^{20-10-1}$* were dissected and stained as mature third instar larvae or adults.

Experiment 6: *Abd-B* knock down from first instar larva onwards.
To knock down *Abd-B* in larval CNS, *elav^{C155}-Gal4; tub-Gal80^{ts}/TM3, Act>GFP* females were crossed with *UAS-Abd-B-RNAi^{12024}* males. Embryos from the crosses were collected over 24 hours at 17ºC, kept for a further 20 hours at 17ºC, then shifted to 29ºC as first instar larva. Individuals of genotype *elav^{C155}-Gal4/+; tub-Gal80^{ts}/UAS-Abd-B-RNAi^{12024}* were dissected and stained as mature third instar larvae or adults.

Experiment 7: *hth* knock down from first instar larva onwards.
To knock down *hth* in larval CNS, *elav^{C155}-Gal4; tub-Gal80^{ts}/TM3, Act>GFP* females were crossed with *UAS-hth-RNAi* (long form) males. Embryos from the crosses were collected over 24 hours at 17ºC, kept for a further 20 hours at 17ºC, then shifted to 29ºC as first instar larvae. Individuals of genotype *elav^{C155}-Gal4/+; tub-Gal80^{ts}/UAS-hth-RNAi* were dissected and stained as mature third instar larvae. We tested RNAi efficiency by labelling Hth and detected low level of protein; thus, the result of this experiment has to be interpreted with caution.

5.6. Immunohistochemistry.

We performed immunohistochemistry procedures as follows. Central nervous systems of 18h. AEL embryos and first instar larvae were dissected in PBS (Phosphate buffer solution) 1x, with sharp tungsten wire (Wolfram, Madrid, Spain) attached to hypodermic needles of 1ml (BD Plastipak, Oxford, UK). These were mounted on polylysined slides and further washes and incubations were performed there. Central nervous systems of second, third instar larvae and

adults were dissected in PBT (PBS with 0.3% Tween20) either with hypodermic needles or with sharp forceps. In these cases, washes and incubations were performed in 1,5 ml eppendorfs or in glass Pyrex ® plates.

For antibody staining, all mentioned tissues were fixed for 20 minutes in 4% formaldehyde (Polysciences #04018) at room temperature (RT). BBT-250 (PBT with 0.1% Bovine serum albumin (BSA) and 250 mM NaCl) was used as blocking agent. We performed 4 washes of 15 minutes each, at RT. Incubation with primary antibodies was done in BBT-250, overnight and at 4ºC. After primary antibody incubation, we blocked with BBT-250 during 1 hour (4 washes of 15 minutes each) at RT, and incubate with secondary antibodies in BBT-250 for 1h 30minutes at dark and at RT. After this, tissues were washed with PBT for 1 hour (4 washes of 15 minutes each) at RT. All incubations and washes were performed in a rotating shaker. When anti-Biotin antibodies were used, after the incubation with the secondary antibody, we washed during 1 hour (4 washes of 15 minutes each) at RT with BBT-250 and incubated with FITC or Texas-Red conjugated Streptavidin antibodies in BBT-250 for 15 minutes at RT. After this, we washed with PBT during 1 hour (4 washes of 15 minutes each) at RT. Alternatively, tissues incubated with anti-Biotin were revealed using the ABC technique (Avidin and Biotinylated horseradish peroxidase macromolecular Complex) following the manufacturer's indications (Vector laboratories), and used diaminobenzidine tetrahydrochloride (DAB) to reveal the staining. Tissues were mounted on a glass slide in Vectashield (Vector, Burlingame, USA) and sealed with nail polish for later microscopy visualization.

Embryos from earlier stages were dechorionated with bleach for 3 minutes and then fixed in heptane/4% formaldehide/methanol for 20 minutes at RT. They were stained using the above-mentioned protocol. Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega, 1985).

The list of the different antibodies used are indicated in Tables 1, 2 and 3.

Table M.1: List of primary antibodies used.

Primary antibodies				
Antibody	Species	Dilution	Reference	Source
Abd-A	rat	1:500	(Macias et al., 1990)	A. Macías, National University of Córdoba, Córdoba, Argentina.
Abd-B	mouse	1:50	#1A2E9	Developmental Studies Hybridoma Bank (DSHB). University of Iowa, Iowa City, USA.
β-galactosidase	chicken	1:200	#ab9361	Abcam antibodies, Cambridge, UK.
β-galactosidase	rabbit	1:2000	#55976	ICN-Cappel Research reagents, Aurora, USA.
β-galactosidase	mouse	1:2000	#40-1a	DSHB. University of Iowa, Iowa City, USA.
Broad-Complex	mouse	1:50	#25E9.D7	DSHB. University of Iowa, Iowa City, USA.
Cas	guinea pig	1:500	(Tsuji et al., 2008)	T.Isshiki, National Institute of Genetics, Mishima, Japan
Cas	rabbit	1:200	(Kambadur et al., 1998)	W.F. Odenwald, National Institute of Health, Bethesda, USA.
Dpn	rabbit	1:500	(Bier et al., 1992)	S.Thor, Linköping University, Linköping, Sweden.
En	mouse	1:50	#4D9	DSHB. University of Iowa, Iowa City, USA.
Eve	mouse	1:50	#3C10	DSHB. University of Iowa, Iowa City, USA.
GFP	chicken	1:200	#AB16901	Millipore, Temecula, USA.
GFP	rabbit	1:200	#A-6455	Invitrogen, Carlsbad, USA.
GFP	mouse	1:100	#11814460	Roche, Nutley, USA.
Grh	rat	1:100	(Baumgardt et al., 2009)	S.Thor, Linköping University, Linköping, Sweden.
Hb	guinea pig	1:200	(Tsuji et al., 2008)	T.Isshiki, National Institute of Genetics, Mishima, Japan.

Hth-RE	guinea pig	1:300	(Corsetti and Azpiazu, 2013)	N. Azpiazu, CBM, Madrid, Spain.
Kni	guinea pig	1:200	(Kosman et al., 1998)	D. Kosman, University of California San Diego, La Jolla, USA.
Kr	guinea pig	1:400	(Tsuji et al., 2008)	T.Isshiki, National Institute of Genetics, Mishima, Japan.
DmLin-29	guinea pig	1:300-500	(Tsuji et al., 2008)	T.Isshiki, National Institute of Genetics, Mishima, Japan.
Lk	rabbit	1:50	(Nassel and Lundquist, 1991)	D. Nässel, Stockholm University, Stockholm, Sweden.
Lk	rabbit	1:100	(Nassel and Lundquist, 1991)	S. Thor, Linköping University, Linköping, Sweden.
Lk	rat	1:1000	(Estacio-Gomez et al., 2013)	F.J. Díaz-Benjumea, CBM, Madrid, Spain.
Nab	rabbit	1:1000	(Terriente Felix et al., 2007)	F.J. Díaz-Benjumea, CBM, Madrid, Spain.
Pdm1	rabbit	1:1000	(Terriente Felix et al., 2007)	F.J. Díaz-Benjumea, CBM, Madrid, Spain.
Tsh	rabbit	1:50	(Ng et al., 1996)	S. Cohen, Institute of Molecular and Cell Biology, Singapore.
Ubx	mouse	1:20	#FP3.38	DSHB. University of Iowa, Iowa City, USA.

Table M.2: List of biotinilated secondary antibodies used.

Biotinilated secondary antibodies				
Antibody	Species	Dilution	Reference	Source
Biotin	chicken	1:500	#703-065-155	Jackson Immunoresearch, West Grove, USA.

Table M.3: List of fluorescent secondary antibodies used.

Fluorescent secondary antibodies				
Antibody	Species	Dilution	Reference	Source
Alexa 488	chicken	1:500	#A-11039	Invitrogen, Carlsbad, USA.
Alexa 488	rabbit	1:500	#A-21026	Invitrogen, Carlsbad, USA.
Alexa 488	rat	1:500	#A-21208	Invitrogen, Carlsbad, USA.
Alexa 488	mouse	1:500	#A-21202	Invitrogen, Carlsbad, USA.
Fluorescein (FITC)-conjugated Streptavidin		1:500	#016-090-084	Jackson Immunoresearch, West Grove, USA.
Alexa 555	rabbit	1:500	#A-31572	Invitrogen, Carlsbad, USA.
Alexa 555	rat	1:500	#A-21434	Invitrogen, Carlsbad, USA.
Alexa 555	mouse	1:500	#A-31570	Invitrogen, Carlsbad, USA.
Alexa 594	rabbit	1:500	#A-21207	Invitrogen, Carlsbad, USA.
Alexa 594	rat	1:500	#A-21209	Invitrogen, Carlsbad, USA.
Alexa 594	mouse	1:500	#A-21203	Invitrogen, Carlsbad, USA.
Texas Red-conjugated Streptavidin		1:500	#016-070-084	Jackson Immunoresearch, West Grove, USA.
Alexa 647	rabbit	1:200	#A-31573	Invitrogen, Carlsbad, USA.
Alexa 647	rat	1:200	#A-21247	Invitrogen, Carlsbad, USA.
Alexa 647	mouse	1:200	#A-31571	Invitrogen, Carlsbad, USA.

5.7. Microscopy Imaging and data acquisition.

All images of central nervous systems are oriented with anterior up, unless otherwise stated. A Leica DMLB microscope connected to a digital camera Nikon CoolPix 990 was used to take the images of conventional microscopy. Zeiss LSM 510, Zeiss LSM 510 META or Zeiss LSM 710 confocal microscopes were used to collect data for all fluorescent images; confocal stacks were merged using Fiji/ImageJ or Adobe Photoshop.

5.8. Statistical methods.

Statistical analysis was performed using Microsoft Excel. Quantifications of observed phenotypes were performed using Student's two-tailed t test, assuming equal variance and *p-value*= 0.01. Data are presented as ABLK cells mean ± standard deviation. An asterisk (*) in the data indicates that there were significant differences compared to *wild-type*.

6. Results

6.1. Description of Leucokinin pattern of expression in late embryonic stage.

The pattern of expression of the leucokinergic system has been described in third instar larvae (Cantera and Nassel, 1992; Herrero et al., 2007; Herrero et al., 2003) and in adult stages (Herrero et al., 2003). Since the main goal of this project was to identify the genetic mechanisms for ABLKs specification, experiments were carried out just after their generation, or alternatively, whenever Lk is first detected, that is at late embryonic stage, at 18 h. after egg laying (AEL). At this stage the embryos are still enclosed within the vitelline membrane, tracheae are visible and filled with air. On the same note, many of the mutant lines studied were embryonic lethal, therefore it became mandatory to describe Lk expression pattern in detail at late embryonic stage.

It is important to mention that we used the expression of Lk as a marker to identify the ABLK neurons, meaning that it would not be possible to distinguish between a situation in which these neurons were not generated and a situation in which they were present but lost the expression of Lk, given that this is an event that occurs during their terminal differentiation.

When we looked at the expression pattern of Lk at 18 h. AEL, it was similar to the one observed at third instar larva, with minor differences. (Fig. R.1A and B). There was one pair of prominent Lk neurons in the brain (LHLKs), a cluster of 2-4 Anterior Lk neurons in each brain lobe (ALKs), and two pairs of Subesophageal Lk neurons in the third subesophageal segment (SELKs), although these latter displayed a fainter Lk signal than the ones detected in

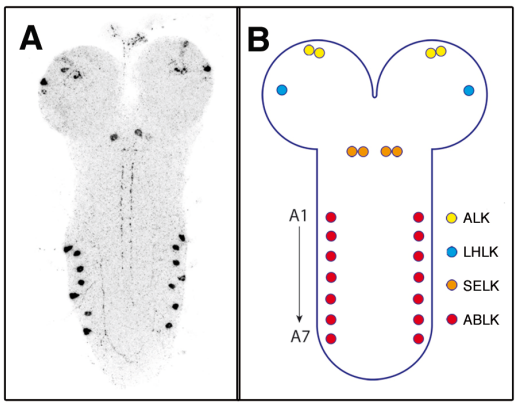


Fig. R.1. Pattern of expression of Leucokinin at 18h after egg laying (AEL) embryo. (**A**). Expression of Leucokinin (Lk) detected by antibody staining. (**B**) Schematic of the leucokinergic system. ALK, Anterior LK; LHLK, lateral horn LK; SELK, subesophageal LK; ABLK, abdominal LK.

third instar larvae. In the abdominal segments we observed one ABLK per hemisegment, at a ventrolateral position, from the first abdominal segment (A1) to the seventh (A7), making a total number of 14 ABLKs per VNC. It is worth saying that the number of ABLKs, at 18 h. AEL, was fixed in all *wild-type* individuals studied (χ =7.00±0.00).

6.2. Identification of the ABLK progenitor neuroblast.

In the study of neural specification of a particular set of neurons it is relevant to know from which lineage those neurons come from and, what other neural types are being generated along with them. Thus, our first aim was to identify the progenitor NB from which ABLKs arise. At the beginning of this project the progenitor of the abdominal leucokinin-expressing cells had never been addressed. However, based on DiI labelling experiments, Schimd et al. (Schmid et al., 1999) described a motoneuron from the NB2-4 lineage that extended to muscle 8 and suggested that it may be the *Drosophila* homolog of the leucokinin cell in *Calliphora*, as reported by Cantera and Nässel (Cantera and Nassel, 1992).

As explained before, work over the last two decades has provided a detailed lineage map of most of the 30 NBs present per hemisegment in the VNC, from late stage 8 to late 11. (Fig. I.6). Each of them can be unambiguously identified by their time of formation, position, the progeny produced and the expression of a unique combination of molecular markers (Bossing et al., 1996; Doe, 1992; Prokop and Technau, 1991; Schmid et al., 1999; Schmidt et al., 1997).

Therefore, to find out which was the progenitor NB of ABLKs, we decided to make use of these molecular markers and check for their expression in the ABLKs at 18 h. AEL. Since it is not known if the expression of most of these markers is maintained in post-mitotic cells, these results need to be taken with caution. Only *gsb* expression has been reported to be maintained by lineage (Buenzow and Holmgren, 1995).

The expression of these molecular markers within the 30 NBs map can be classified within domains (Doe lab website: http://www.neuro.uoregon.edu/doelab/nbmap.html). *mirror (mirr)-lacZ* is present in the NBs from anterior rows, mostly in row 1, 2 and 3 NBs. *wingless* (*wg)-lacZ* is specifically expressed in row 5 NBs, whereas *gsb-distal* does so in NBs

from rows 5-6 and in NBs 7-1 and 7-3. Engrailed (En) is expressed in the NBs of the most posterior rows, that is, all NBs of rows 6-7 and in NB1-2 (Fig. R.2). The rest of the molecular markers are expressed in individual or in scattered groups of NBs, with the exception of the temporal gene *cas,* also known as *ming* (*ming-lacZ*), which is present in 19 out of the 30 NBs, and the temporal switching factor *svp* (*svp-lacZ*), which is expressed in almost all NBs by late stage 11.

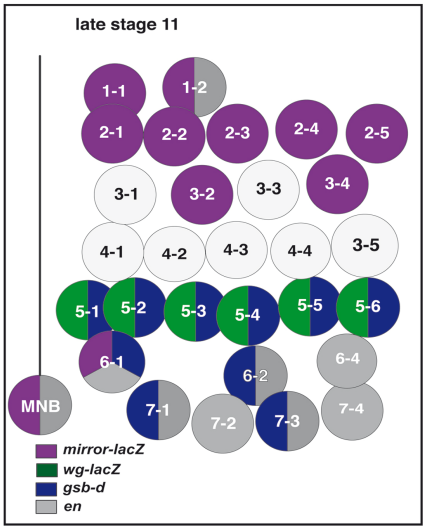


Fig. R.2. Neuroblast map of a late stage 11 embryo. This cartoon represents the 30 neuroblasts (NBs) found in each hemisegment of the ventral nerve cord according to the expression of the following molecular markers: *mirror-lacZ* (purple), *wg-lacZ* (green), *gsb-d* (blue) and *engrailed* (grey). NBs that do not express any of these molecular markers are coloured in white. The midline is represented by a vertical line on the left, anterior is up. Modified from (Doe, 1992)

So far, only two markers have been described to be specifically expressed in a NB or in a set of NBs, and in their progeny, namely: *eagle (eg-lacZ)* and *ladybird early* fragment K driving *lacZ (lbe (K)-lacZ). eg* is expressed in NBs 2-4, 3-3, 6-4 and 7-3 at their corresponding times of delamination and in their progeny (Berger et al., 2005; Dittrich et al., 1997; Higashijima et al., 1996; Isshiki et al., 2001; Tsuji et al., 2008). Likewise, *lbe(K)-lacZ* is specifically expressed in NB5-6 and its progeny (Baumgardt et al., 2009; De Graeve et al., 2004; Karlsson et al., 2010).

We found that ABLK cells expressed the following lines: *gsb distal-lacZ (gsb-d-lacZ), hkb-lacZ, unplugged-lacZ (unpg-lacZ)* and *wg-lacZ* (Fig. R.3A-H). We did not detect co-expression between ABLKs and the following markers: *eg-Gal4* (data not shown), En, Even-skipped (Eve), *lbe (K)-lacZ* and *mirr-lacZ* (Fig. R.3A-H). The presence of *wg-lacZ* and *gsb-lacZ* in ABLKs pointed towards a row 5 NB. On the other hand, the lack of coexpression with *lbe (K)-lacZ* excluded NB5-6. Among the rest of row 5 NBs, only NB5-5 expressed both *hkb-lacZ* and *unpg-lacZ*. These findings together with the lateral position of ABLKs, strongly supported NB5-5 as the progenitor of ABLK cells, yet, only *gsb* expression in NBs is known to be maintained in

their progeny. Therefore, we cannot rule out the possibility that the combination of markers expressed by the ABLK progenitor NB changes over time. Therefore, a given gene could be expressed in the NB but absent in the postmitotic ABLK and vice versa, a gene expression could be gained later in development and, appear specifically in the neuron but not in its progenitor NB.

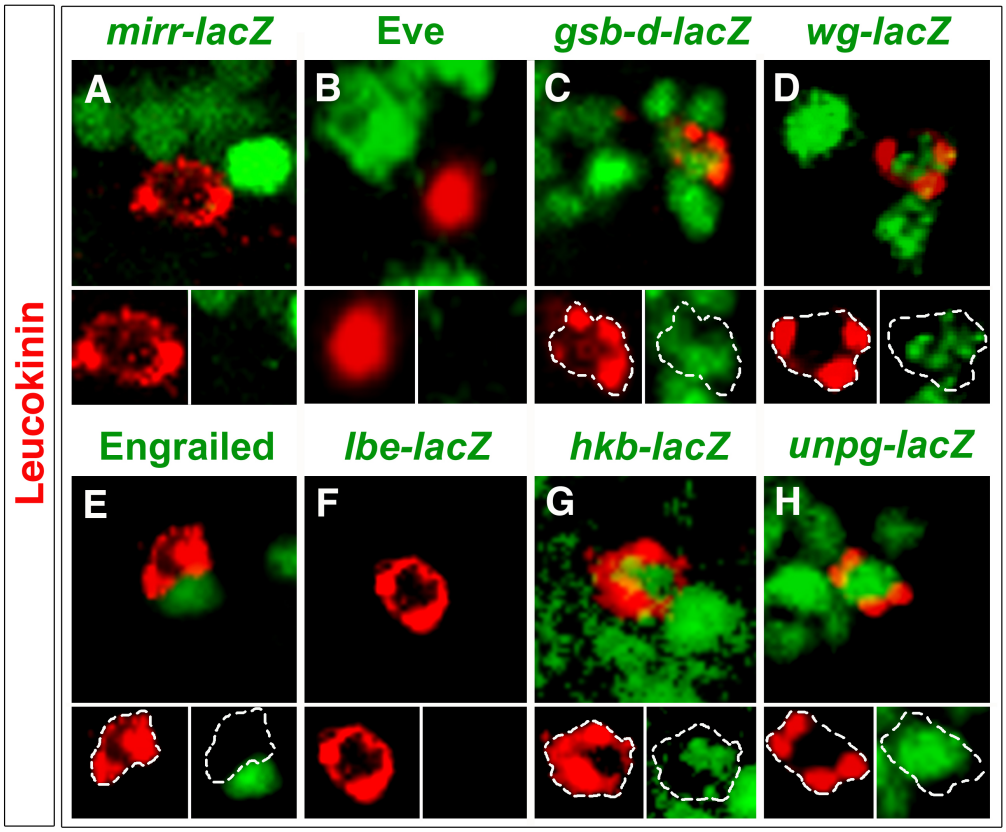


Fig. R.3. Identification of ABLK progenitor neuroblast. (**A-H**) Expression of Lk (red) and *mirr-lacZ* (**A**), Eve (**B**), *gsbd-lacZ* (**C**), *wg-lacZ* (**D**), En (**E**), *lbe-lacZ* (**F**), *hkb-lacZ* (**G**) and *unpg-lacZ* (**H**) (green) in the ventral nerve cord of *Drosophila* 18h AEL embryo. ABLKs coexpress *gsbd-lacZ*, *wg-lacZ* and *unpg-lacZ.* Dashed outline represents an ABLK. Separate channels are shown at the bottom of the figure.

To further confirm our hypothesis that NB5-5 was the progenitor of ABLKs, we performed a lineage tracing experiment of row 5 NBs. Since *gsb-Gal4* lines were not available, we used a *wg-Gal4* line to confirm that the ABLKs progenitor NB belonged to row five. We tracked the lineage of cells expressing *wg* at late stage 11 (see experiment 1 in Materials and

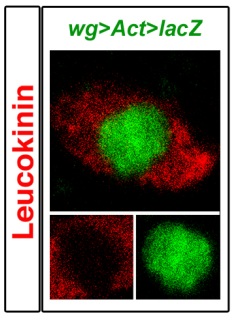


Fig. R.4. Labelling of *wg-Gal4* expressing cells at early stages of development. Expression of Lk (red) and *β-galactosidase* (*act5C>lacZ*; green) in lineage-tracing experiment (see main text and Materials and methods for details). ABLKs arise from a *wg*-expressing NB. Separate channels are shown at the bottom of the figure.

Methods), by inducing early permanent expression of *β-galactosidase* in all row five NBs and their progeny, NB5-5 included. We found that ABLKs expressed β-galactosidase, which confirmed that ABLKs were born from a row 5 NB (Fig. R.4).

Next, in the absence of markers that allowed us to detect ABLKs at early times, we tracked the expression of *unpg-lacZ* in NB5-5 from its initial activation at late stage 11 to 18 h. AEL, when ABLKs were first detected. Using this marker, we observed that the expanding cluster of cells expressing *unpg-lacZ* could be followed up into first instar larva, when one of the cells coexpressed Lk (Fig. R.5A-E).

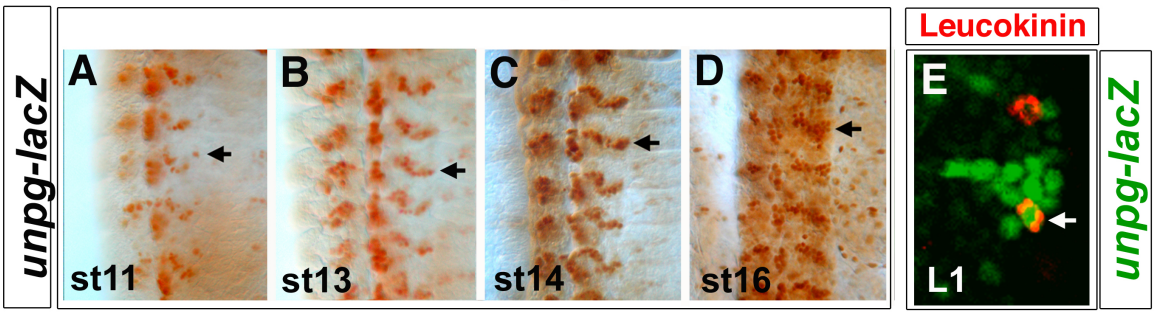


Fig. R.5. Expression of *unpg-lacZ* from stage 11 to first instar larvae. Expression of *unpg-lacZ* in embryos of stage 11 (**A**), 13 (**B**), 14 (**C**) and 16 (**D**). Black arrows point to NB5-5 and its putative progeny. (**E**) Expression of *unpg-lacZ* (green) in first instar larvae and Lk (red). White arrow indicates an ABLK. ABLKs belong to the *unpg-lacZ*-expressing progeny.

These results, together with the coexpression of molecular markers and the *wg-lacZ* lineage tracing experiment, allowed us to conclude that ABLKs are most probably generated by NB5-5 (Fig. R.6).

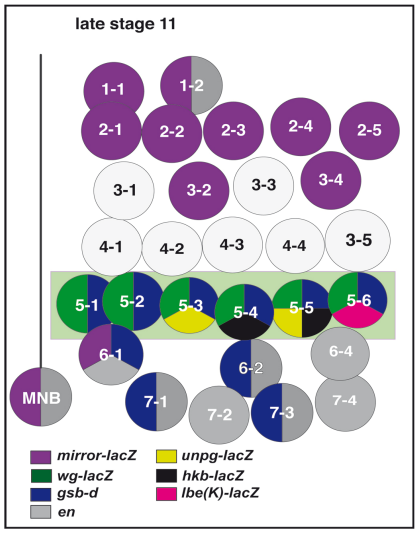


Fig. R.6. Summary. Cartoon summarizing the results. It is shown a NB map present in a right hemisegment of a late stage 11 embryo coloured according to the pattern of expression of the following molecular markers: *mirror-lacZ* (purple), *wg-lacZ* (green), *gsb-d* (blue) and *engrailed* (grey). NBs that do not express any of these molecular markers are coloured in white. The midline is represented by a vertical line on the left, anterior is up. Only NBs from row 5 (green framed) are described according to the expression of *unpg-lacZ* (yellow), *hkb-lacZ* (black) and *lbe (K)-lacZ* (pink). These findings indicate that ABLKs are most probably generated by NB5-5.

NB5-5 delaminates at the fifth wave of delamination, at late stage 11 (Doe, 1992). Identification of NB5-5 and its progeny has been problematic in previous clonal analysis of embryonic NBs by *in vivo* DiI (a lipophilic fluorescent tracer) labelling. Indeed, work from Technau's lab was not able to assign a lineage to NB5-5 (Schmidt et al., 1997). Later on, studies from Doe's lab managed to generate four NB5-5 clones, three of them were thoracic and one was abdominal (Schmid et al., 1999). This abdominal clone was assayed at stage 17 and comprised between 8 and 11 cells, including local interneurons and neurosecretory cells. They described a ventral neurosecretory cell that was present in both thoracic and abdominal clones. It extended two bifurcating projections laterally before joining the transverse nerve (TN). This putative neurosecretory cell was similar to the description of the *Manduca* Va neurosecretory cell in shape and ganglionic position (Carr and Taghert, 1988a, b; O'Brien and Taghert, 1998). Its location was also similar to that of the medial Crustacean cardioactive peptide (CCAP)-positive cell previously observed in *Drosophila* and *Manduca* (Broadie et al., 1990; Tublitz and Sylwester, 1990). Likewise, since its mature endings might form postembryonically, they did not rule out the possibilty that this cell was a TN motoneuron, as Schmidt et al. (Schmidt et al., 1997) had proposed.

The fact that the analysis of the axonal projections, from the clonal analysis, was performed between stages 15 and 17 might explain the diverse identities proposed for this putative neurosecretory cell, and therefore, it is not surprising that none of the axonal projections described there coincided with the axonal projections of the ABLKs, which were

characterized in third instar larvae and proposed to leave the CNS through the Segmental Nerve a (SNa) (Cantera and Nassel, 1992; Johansen et al., 1989). Additionally, ABLKs are likely to belong to a lateral cluster of four dimm-expressing cells described in abdominal segments and referred to as the peptidergic lateral cluster (PLC) (Miguel-Aliaga et al., 2004; Park et al., 2008). ABLK cells extend along the SNa, project across muscle eight, then follow the intersegmental nerve (ISN) out, and terminate on the alary muscles (Cantera and Nassel, 1992; Landgraf and Thor, 2006).

6.3. Identification of the temporal sequence of NB5-5.

Although it has been reported that NBs sequentially express a series of transcription factors that provide temporal identities to the different neurons generated, not all NBs pass through the complete temporal series. Thus, we wanted to know which temporal factors NB5-5 expressed.

NB5-5 delaminates in the fifth wave of delamination, at late stage 11. We stained embryos of this stage with antibodies against the five known temporal genes. We made use of the *gsb-lacZ* line to label NBs of rows 5 and 6, to most accurately identify NB5-5 from late stage 11 to 13. The NB marker Deadpan (Dpn) was employed to distinguish between the progenitors and their progeny, given that both of them expressed the reporter β-*galactosidase*. The increasing number of cells that are produced towards later stages of development made difficult to unambiguously identify NB5-5 further on.

At late stage 11 NB5-5 did not express neither Hb nor Kr, but we detected expression of Pdm. However, this was rapidly lost by early stage 12. Cas expression started at stage 12 and continued till stage 13, when Grh was first seen (Fig. R.7A-F).

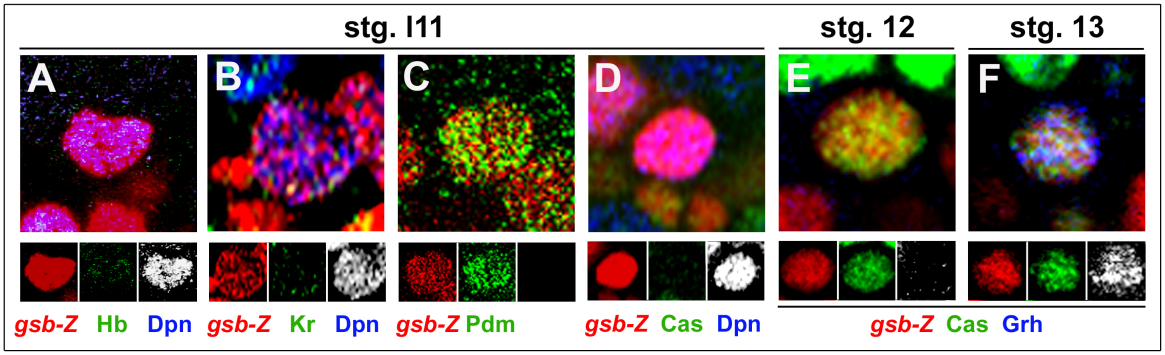


Fig. R.7. Sequence of expression of temporal factors in NB5-5. (**A-F**) Expression of *gsb-lacZ* (red), Deadpan (A, B, D; blue) and Hb (A), Kr (B), Pdm (C), Cas (D-F) (green) and Grh (E, F; blue) in NB5-5 at late stage 11 (A-D), stage 12 (E) and stage 13 (F). NB5-5 expresses Pdm (C) when it delaminates, but not Hb nor Kr (A-B). NB5-5 is positive for Cas at stage 12 (E) and it coexpresses both Cas and Grh at stage 13 (F). The anti-Deadpan (Dpn) is employed to label NBs. NB5-5 is identified by expression of *gsb-lacZ* and position. The separate channels are shown at the bottom of each figure. For clarity the blue channel is shown in white.

In conclusion, we found that NB5-5 started its temporal series with a Pdm window, followed by a Cas window and later by a Cas/Grh temporal window. We cannot know how many divisions the NBs keep each of the temporal windows, nor how many neurons or glial cells are generated in each of them. As individual Pdm-temporal windows do not typically extend further than a single division (Baumgardt et al., 2009; Bhat et al., 1995; Grosskortenhaus et al., 2006; Tran and Doe, 2008; Tsuji et al., 2008), we consider that the NB will be Cas positive during its second round of division. On the other hand, the extension of the Cas window is quite variable in different NBs (Doe, 1992).

6.4. Identification of the temporal window of ABLK neurons.

Next, we investigated the temporal window in which ABLKs were generated. For that, we performed loss- and gain-of-function experiments for all the temporal genes known.

In agreement with the observation presented above that NB5-5 did not express early temporal genes, we observed no changes in ABLKs number in *hb (elav-Gal4>UAS-hb* RNAi *UAS-Dicer2* χ=7.00±0.00), and a slight decrease in *Kr* (*Kr*^{1} *Kr*^{CD} χ=4.82±1.47*) 18h. AEL embryos. On the other hand, despite the fact that NB5-5 was positive for Pdm when it delaminated, we did not find phenotype in *pdm (Df (2L) ED773,* χ=6.85±0.46). Lastly, all

ABLKs dissapeared in *cas* (*cas*^{D1} χ =0.00±0.00*) and their number was severely reduced in *grh* (*grh*^{370} χ =2.60±1.73*). These results pointed towards a Cas/Grh temporal window as the one in which ABLKs were produced (Fig. R.8A-H).

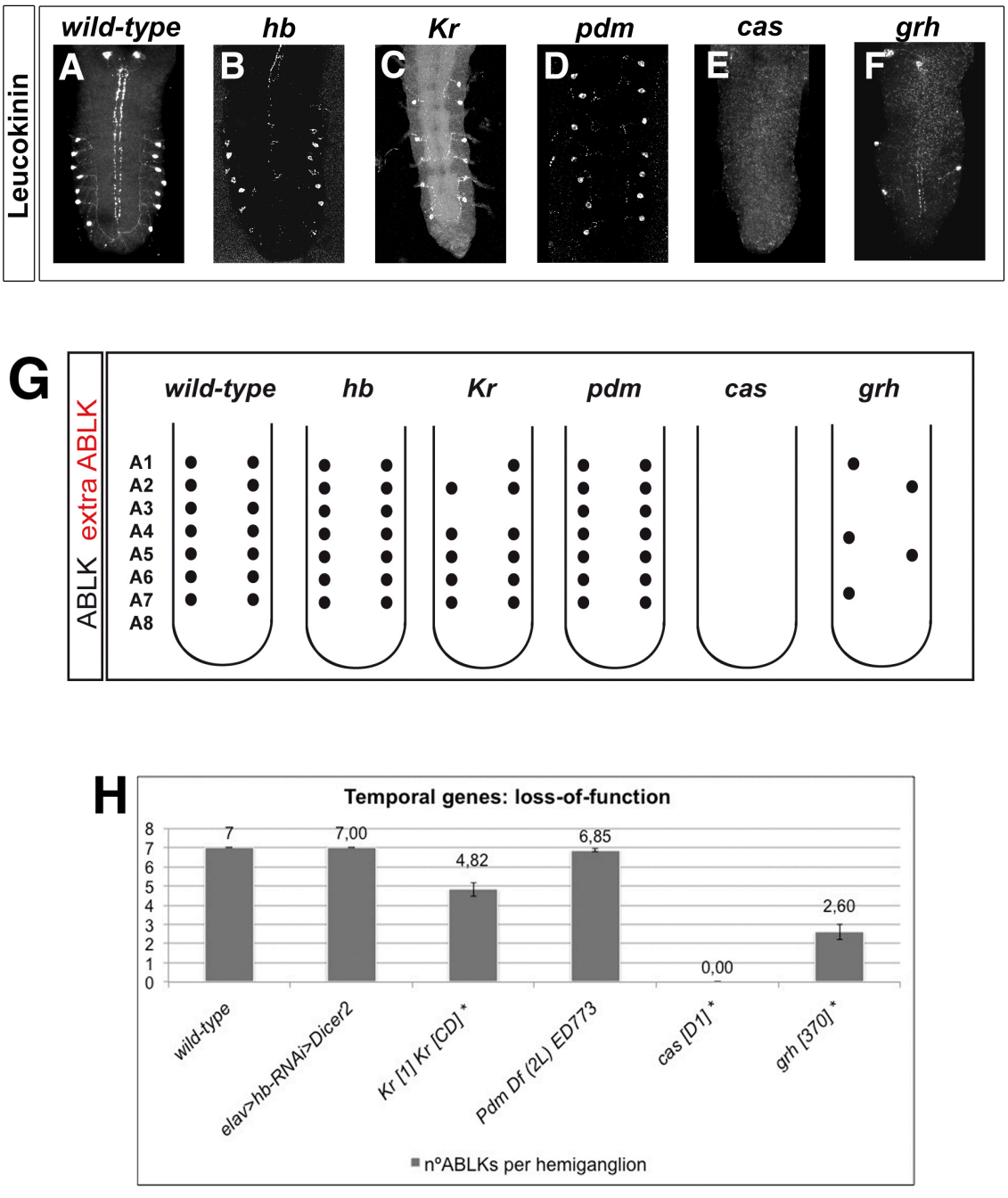


Fig. R.8. The temporal sequence of ABLKs. (**A-F**) Expression of Lk in *wild-type* (A), *elav-*

Gal4>UAS-hb RNAi UAS-Dicer2 (B), *Kr*^{1} *Kr*^{CD} (C), *Df(2L)ED773* (*pdm*$^{-}$) (D), *cas*^{D1} (E) and *grh*^{370} (F) in ventral nerve cords of 18h AEL embryos. ABLKs are strongly reduced in *cas*^{D1} and *grh*^{370} embryos (E-F). (**G**) Cartoons summarizing the phenotypes observed. Black circles represent *wild-type* ABLKs and red ones represent extra ABLKs. Abdominal segments are numbered on the left. (**H**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type*.

We also observed that ABLKs were duplicated in some hemisegments in *svp* mutants (*svp*^{1} χ =7.86±0.79*, Fig. R.9B, E and F). Svp presents a dynamic pattern of expression within the nervous system; it is first expressed in NBs at early times of development, around stage 10, when it represses *hb* activity. Consequently, the number of cells born within a Hb-expressing window, that is, early-born neurons, increases when *svp* is mutated, as a result of an extended Hb temporal fate; whereas the number of late-born neurons decreases (Kanai et al., 2005; Mettler et al., 2006). Then, *svp* expression disappears from NBs and reappears in some NBs around stages 14-15. The function of this second wave of *svp* is hitherto unknown (Benito-Sipos et al., 2011; Kohwi et al., 2011; Tsuji et al., 2008). However, it has been shown that in NB5-6T lineage, this second pulse of expression of *svp* is necessary to subdivide the Cas/Grh temporal window that produces the Apterous (Ap) 1-4 cluster cells. Specifically, *svp* is expressed in a very discrete fashion in Ap2/3 interneurons to ensure their proper specification against the Ap1 and Ap4 cell fates (Benito-Sipos et al., 2011). Yet, it is not clear if this subtemporal function of the second pulse of *svp* occurs in other lineages. The authors did not observe global changes in the known *svp*-target genes expression levels in the CNS, so if *svp* performs this role in other lineages, it might involve different targets and be context-dependent.

Since the results presented above did not indicate that ABLKs came from an Hb positive NB, we wondered whether *svp* was performing this late function in ABLKs specification. First, we found that ABLKs expressed *svp-lacZ* (in 86% of ABLKs scored, n=42, Fig. R.9D). We assumed that *svp* was expressed during its second wave in the NB5-5, while ABLKs were generated. Next, the results we obtained upon *svp* misexpression also pointed towards this idea, we observed ocasional duplications of ABLKs in some hemisegments, yet these changes were not statistically significant at 99% level of confidence. (*wor-Gal4>UAS-svp:* χ =7.41±1.05 and *elav-Gal4>UAS-svp:* χ =7.92±1.24, Fig. R.9C, E and F). These results were similar to the ones obtained for NB5-6T. There, *svp* misexpression generated duplications in Ap2/3

neurons, but they did not observe overall changes in the number of Ap cluster neurons. Thus, we concluded that most likely, the function of Svp in NB5-5 lineage was to subdivide the wide Cas/Grh window, as it has been described elsewhere.

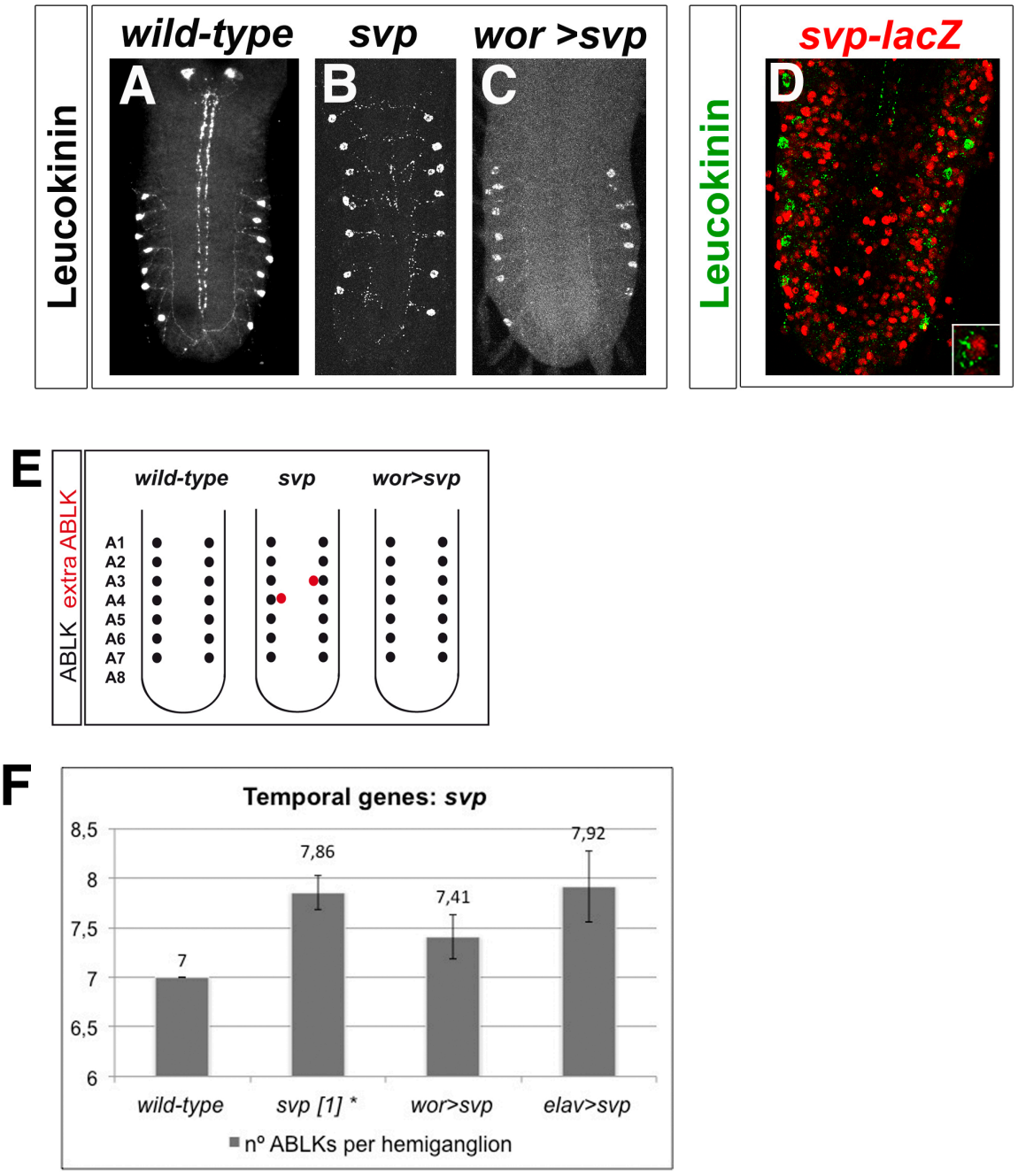


Fig. R.9. The role of *svp* in ABLKs specification. (**A-C**) Expression of Lk in *wild-type* (A), *svp*^{1} (B) and *wor-Gal4>UAS-svp* (C) in ventral nerve cords of 18h AEL embryos. There are duplications of ABLKs in some hemisegments in *svp*^{1} (B), whereas misexpression of *svp* does

not cause a significant phenotype (C). (**D**) Coexpression of *svp-lacZ* (green) and Lk (red) in a 18h AEL embryo. A higher magnification view of one hemisegment is shown at the bottom of the figure. (**E**) Cartoons summarizing the phenotypes observed. (**F**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type*.

The findings presented above strongly suggested that ABLKs were generated in a Cas/Grh temporal window; therefore, we performed coexpression experiments and found that Cas and *grh* (*grh-lacZ)* were expressed in ABLKs at 18h. AEL (Fig. R.10 A and B).

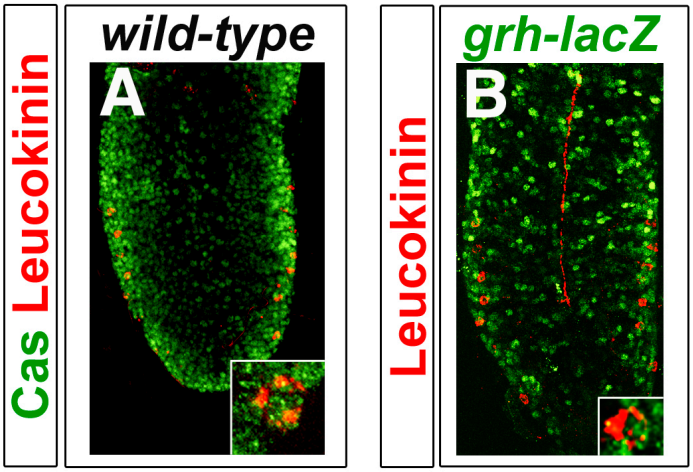


Fig. R.10. The temporal genes *cas* and *grh* are expresed in ABLKs. (**A**) Expression of Lk (red) and Cas (green) in *wild-type*, and (**B**) expression of Lk (red) and β-galactosidase (green) in *grh-lacZ* 18h AEL embryos. A magnification of an ABLK is shown in the insets.

To further confirm these results, we overexpressed the temporal genes with neuroblast drivers: *worniu-Gal4 (wor-Gal4)* or *inscuteable-Gal4 (ins-Gal4),* and with the panneuronal driver *elav-Gal4*. We found no changes in the pattern of expression of ABLKs when misexpressing *Kr* (*insG4>Kr* χ =6.47±1.78) or *pdm* (*wor>pdm* χ =7.00±0.00 and *elav>pdm* χ =7.00±0.00, Fig. R11K).

Consistent with ABLKs being generated in a Cas window, its misexpression with both *ins-Gal4* and *elav-Gal4* produced ectopic ABLKs (χ =8.11±0.83* and χ =16.38±2.24*, respectively), although the strongest phenotype was detected with the latter (Fig. R11B, J and K). These extra ABLKs appeared in clusters of 2-3 cells, but it was not possible to distinguish the bona fide ABLKs from the ectopic ones. To confirm that these extra ABLKs belonged to the NB5-5 lineage, we analysed the expression of *gsb* and the NB5-6 marker *lbe (gsb-lacZ, elav-*

Gal4> UAS-cas; and *lbe-(K)-lacZ, elav-Gal4> UAS-cas,* respectively*).* We observed that all ABLKs found upon *cas* misexpression expressed *gsb-lacZ* (Fig. R.11C) but not *lbe* (data not shown). These results confirmed that these extra ABLKs were produced by a row 5 or 6 NB. Also, since all of these neurons appeared clustered, we concluded that they were most probably generated by NB5-5.

Similarly, we overexpressed *UAS-grh* with both *wor-Gal4* (χ =7.00±0.00), and *elav-Gal4* (χ =6.83±0.51) and found that the number of ABLKs was not significantly altered (Fig. R.11D, J and K). This suggested that *grh* was not enough by itself to produce extra ABLKs. We thought that the appearance of extra ABLKs would require the combination of both *cas* and *grh.* However, when we misexpressed these two temporal genes (*elav-Gal4> UAS-grh UAS-cas*), we did not find more extra ABLKs than misexpressing *cas* alone (χ =13.75±1.04* compared to χ =16.38±2.24*, Fig. R.11E, J and K). Considering that Cas activates *grh* (Baumgardt et al., 2009), this result is not unexpected. However, when we overexpressed Cas in a *grh* mutant background, the number of ABLKs increased more than overexpressing Cas alone. (*grh^{IM}, elav-Gal4>UAS-cas* χ =21.42±4.02*, Fig. R.11F, J and K). This can be explained due to the ability of Grh to repress *cas* activity (Baumgardt et al., 2009).

On the other hand, it has been recently found that the basic helix-loop-helix (bHLH) protein Dimmed (Dimm), which regulates neuroendocrine cell differentiation (Allan et al., 2005; Hamanaka et al., 2010; Hewes et al., 2003), can act with *grh* to trigger ectopic neuropeptide expression (Baumgardt et al., 2009). Dimm is expressed in the ABLKs (Hewes et al., 2006; Park et al., 2008), and their number is reduced in *dimm* mutants (*dimm^{rev8}* χ =4.90±2.08*, Fig. R.11K). Misexpression of *dimm* alone had no effect upon the number of ABLKs (χ =7.00±0.00, Fig. R.11G, J and K), although we were able to detect new LK-expressing cells near the midline. (Fig. R.11G). They showed weak immunostaining and were localized between abdominal segments A2 to A5 (χ =2.50±0.65). However, we do not consider them as ABLKs, since their localization is extremely different from the bona fide ones. Accordingly, we found that co-misexpression of *grh* and *dimm* was enough to generate ectopic ABLKs that appeared in clusters of 2-3 cells (*elav-Gal>UAS-dimm, UAS-grh,* χ =10.88±1.36*). (Fig. R.11H, J and K).

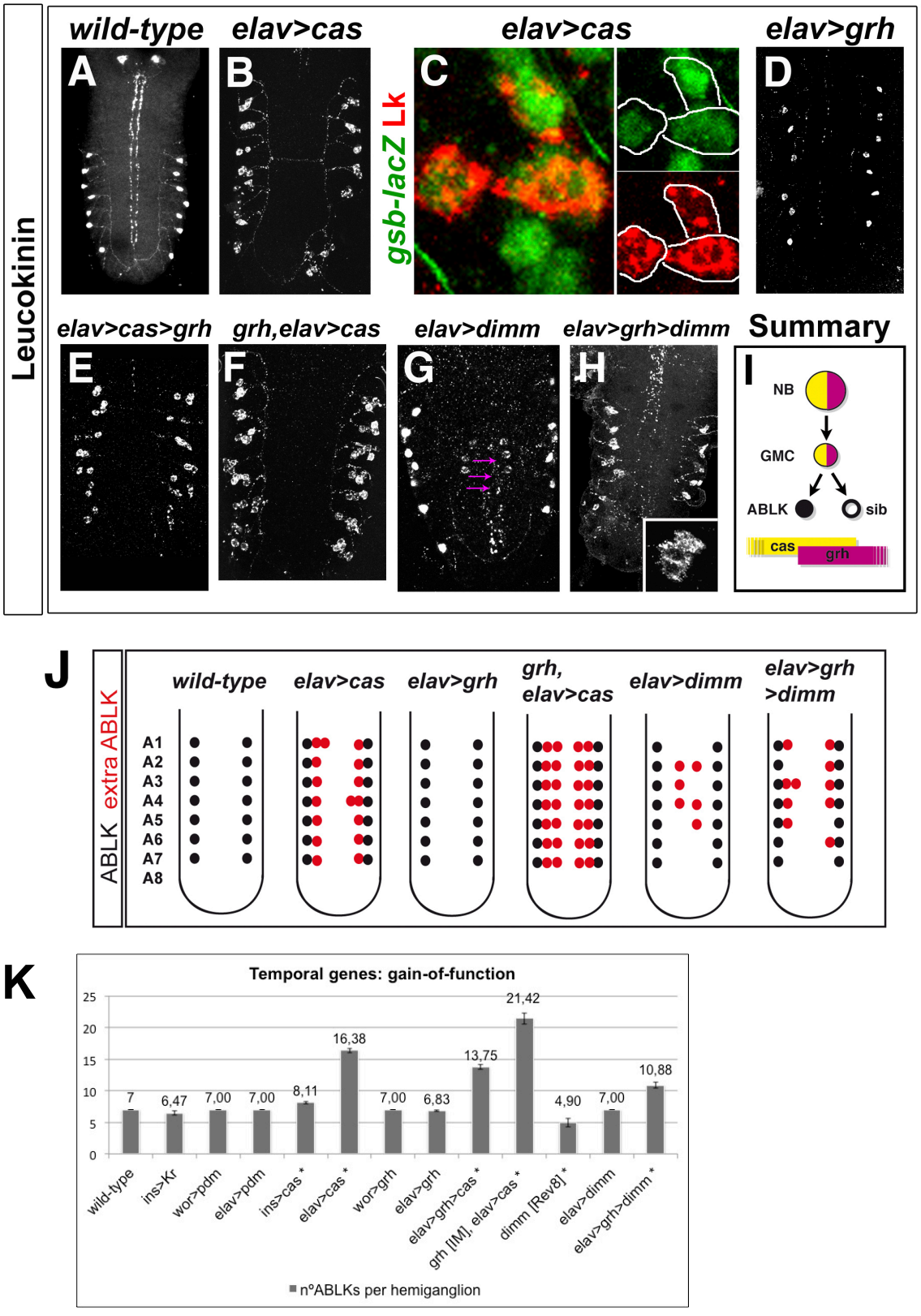


Fig. R.11. ABLKs are specified in a Cas/Grh temporal window. (**A-B**) Expression of Lk in

wild-type (A) and *elav-Gal4>UAS-cas* (B). (**C**) High magnification of single hemineuromeres (outlined) in *elav-Gal4>UAS-cas* showing the expression of Lk (red) and *gsb-lacZ* (green). (**D-F**) Expression of Lk in *elav-Gal4>UAS-grh* (D), *elav-Gal4>UAS-cas UAS-grh* (E) and *grh^{IM}, elav-Gal4>UAS-cas* (F). (**G**) Expression of Lk in *elav-Gal4>UAS-dimm*. Pink arrows show Lk-expressing neurons near the midline in abdominal segments. (**H**) Expression of Lk in *elav-Gal4>UAS-grh UAS-dimm*. A magnification of an ABLK is depicted in the inset. Extra ABLKs appeared upon misexpression of *cas* (B, E-F) and *grh* together with *dimm* (H). All the ventral nerve cords of A-H figures are of 18h AEL embryos. (**I**) Cartoon summarizing these results. (**J**) Cartoons summarizing the phenotypes observed. (**K**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type*.

Lastly, we found ectopic ABLKs, in clusters of 2-3 cells, when misexpressing *hb* with *elav-Gal4* (χ=12.08±1.61*, Fig. R.12C, F and G), whereas there was a significant decrease in the number of ABLKs when misexpressed with *wor-Gal4* (χ=3.44±2.57*, Fig. R.12B, F and G). It has been reported that continuous expression of *hb* in the NB does not allow for the progression of the temporal series and the NB only generates *hb*-expressing neurons, consequently, later-born neurons do not appear (Isshiki et al., 2001; Maurange, 2012). This will explain the decrease of ABLKs found when misexpressing *hb* in the NB with *wor-Gal4*. About the extra ABLKs found in *elav-Gal>UAS-hb* experiment, our results did not suggest that *hb* was involved in the specification of ABLKs. To figure out the origin of these extra ABLKs, we wondered if *cas* was involved in their specification. First, we confirmed that all of them expressed Cas (Fig. R.12D). Second, we misexpressed *hb* in individuals' mutant for *cas* (*cas $^{D1/4}$, elav-Gal4>UAS-hb*), and although the pattern of expression was not completely *wild-type*, some hemisegments lacked ABLKs and others had duplications, there was a partial rescue of ABLKs (χ=7.71±2.05, Fig. R.12E, F and G). It has been described that Hb and Cas share DNA-binding sites. The Cas consensus DNA-binding sequence matches 9 bp out of 10 bp for the reported Hb sites (Stanojevic et al., 1989; Treisman and Desplan, 1989). In the CNS, Hb and Cas repress the activity of Pdm at different times of development, which ensures the proper progession of the temporal series (Kambadur et al., 1998). Our results indicated that, in the absence of Cas, Hb was able to activate, at least, the Cas targets that lead to the activation of the ABLK fate code.

The results presented here confirm that ABLKs are generated in a Cas/Grh temporal window. Consequently, ABLKs are lost or strongly reduced in the loss-of-function experiments of these genes. Additionally, we assume that continued expression of Cas in the

NB is able to maintain the ABLK temporal window further, and thus allows the production of ectopic ABLKs (Fig. R.11I).

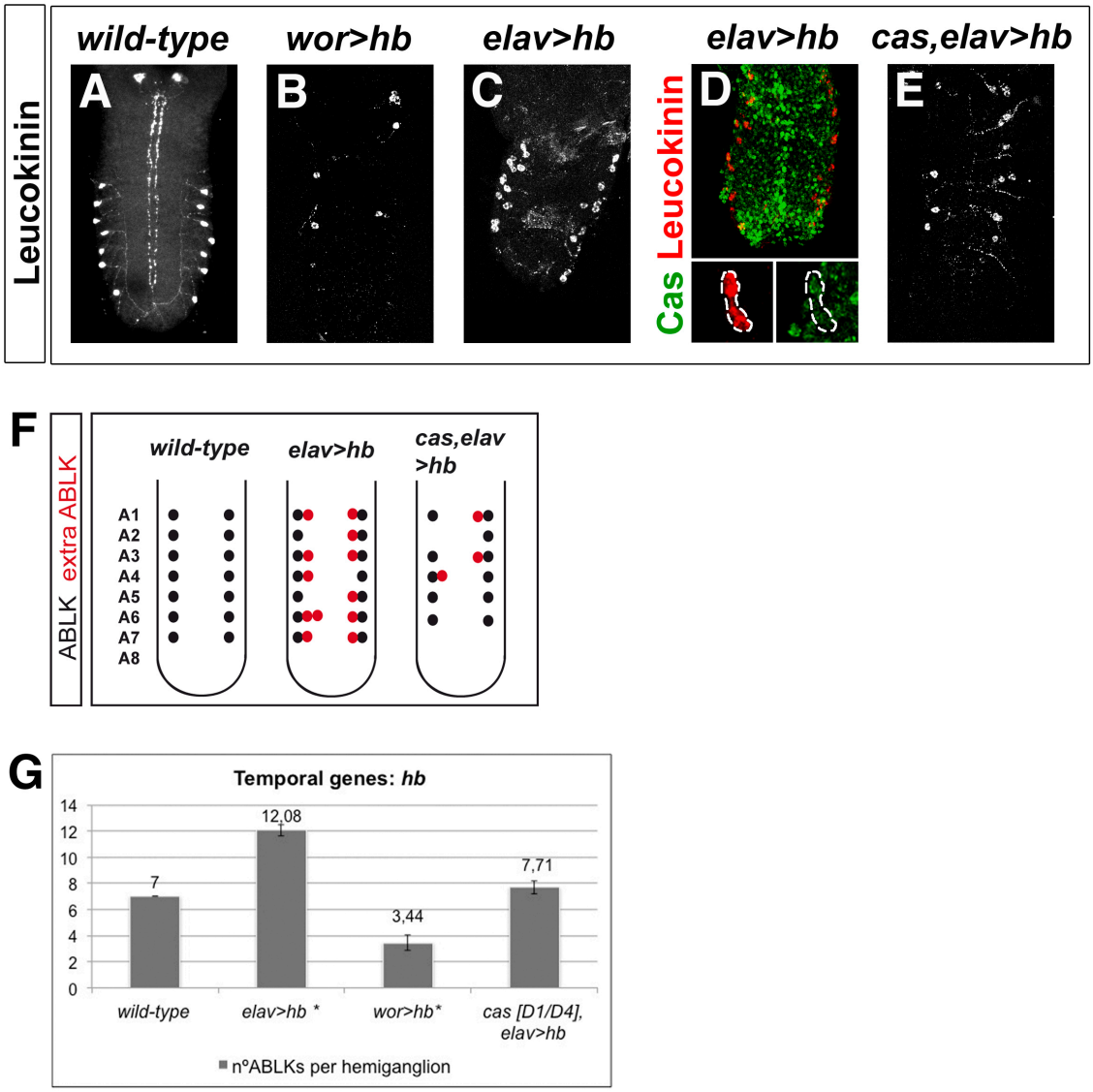


Fig. R.12. Role of the temporal gene *hb* in the specification of ABLKs. (**A-C**) Expression of Lk in *wild-type* (A), *wor-Gal4>UAS-hb* (B) and *elav-Gal4>UAS-hb* (C). The pattern of ABLKs is significant reduced in *wor-Gal4>UAS-hb* (B), whereas it increases in *elav-Gal4>UAS-cas* (C). (**D**) Expression of Lk (red) and Cas (green) in *elav-Gal4>UAS-hb.* Ectopic ABLKs coexpress Cas, in the same way as the bona finde ones. A magnification of duplicated ABLKs are shown in the insets, in separate channels. (**E**) Expression of Lk in $cas^{D1/D4}$, *elav-Gal4>UAS-hb.* Misexpression of *hb* is able to rescue the lack of ABLKs found in $cas^{D1/D4}$. All the ventral nerve cords of A-E figures are of 18h AEL embryos. (**F**) Cartoons summarizing the phenotypes observed. (**G**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type*.

6.5. Role of the Notch signalling pathway in the specification of the ABLK fate.

Neuroblasts divide asymmetrically, self-renew and produce a smaller daughter cell called Ganglion Mother Cell (GMC). GMCs typically divide only once to generate two postmitotic sibling cells, which differentiate into neurons and/or glial cells, or die by apoptosis (Doe, 2008; Knoblich, 2008; Skeath and Thor, 2003; Udolph, 2012). In most, if not in all cases, the GMC division is asymmetric, and the two postmitotic sibling cells acquire distinct cell fates that are normally termed as A and B cells.

As it has been described that, in many cases, the sibling cell of a neuron dies by apoptosis, we wondered about the identity of the ABLK sibling cell. For that, we first repressed programmed cell death (PCD) by overexpressing the baculovirus caspase inhibitor p35 (*elav-Gal4>UAS-p35*) (Hay et al., 1994). We observed that ABLKs were duplicated in many hemisegments (χ=11.50±1.84*, Fig. R.13B, K and L). We obtained a similar result in individuals homozygous for the deficiency *Df(3L)H99* (χ=11.17±2.04*, Fig. R.13C, K and L); deletion of this region abolishes PCD completely (White et al., 1994). These findings suggested that the ABLK had a sibling cell that usually died by apoptosis, but it could assume the ABLK fate if PCD was prevented.

In the CNS, Notch signalling pathway provides cellular diversity acting to endow differential binary cell fate specification programms to sibling cells generated in asymmetric cell divisions. When a GMC divides, one of the sibling cells activates Notch signalling (fate A), whereas the other sibling does not (fate B), and only develops correctly if Notch signalling is not functional. In addition, the activation of Notch signalling in only one of the sibling cells depends on the correct asymmetric partition of the intrinsic cell-fate determinant Numb. Numb physically interacts with Notch and behaves as a negative regulator of the pathway. Therefore, the sibling cell that differentiates into fate A does not possess Numb and activates Notch signalling pathway (Udolph, 2012). Moreover, this pathway has also been described to promote PCD in the progeny, in a lineage specifc manner (Karcavich and Doe, 2005; Lundell et al., 2003; Novotny et al., 2002).

We then wondered about the role of the Notch signalling pathway in the specification of the ABLK. First, we inactivated the pathway using mutant lines for *sanpodo* (*spdo*). *spdo*

encodes a novel transmembrane protein that is required for N signalling during asymmetric cell divisions, but permits normal N function during early neurogenesis (Babaoglan et al., 2009; Dye et al., 1998; O'Connor-Giles and Skeath, 2003; Skeath and Doe, 1998). This allowed the examination of N signalling at later stages of development. We observed that in this mutant the number of ABLKs was strongly reduced (*spdo*^{G104} χ =1.00±0.00*, Fig. R.13D, K and L). We obtained a similar result in *mastermind* (*mam*^{GA345}) mutant (χ =0.00±0.00*, Fig. R.13E, K and L). Mam is a transcriptional co-factor that interacts with the Notch intracellular domain (Petcherski and Kimble, 2000a, b). Conversely, both overexpression of a constitutively active form of Notch (*elav-Gal4>UAS-Nintra*) and loss of *numb (numb*^{1}*)*, produced an increase in the number of ABLKs (χ =13.85±0.38*, and χ =11.52±2.14*, respectively. Fig. R.13F-G, K and L). Together, these results indicated that N signalling was required to activate the ABLK fate.

The findings presented so far suggested that first, ABLKs did have a sibling cell that died by apoptosis, but otherwise was able to adopt an ABLK fate and, second, that N signalling pathway was ON in the ABLKs and OFF in the sibling cell. Therefore, we considered two different scenarios: N signalling could be required to activate the ABLK fate and this, in turn, would be enough to repress apoptosis, or alternatively, N signalling would not have an instructive role in ABLK specification and it would only act to repress PCD. We favour the latter explanation, since the solely removal of apoptosis was able to rescue the sibling cell, suggesting that this cell had complete potential to develop as an ABLK, but it failed to do so because apoptosis was not repressed, in this case, by Notch signalling pathway.

To obtain further data that allowed us to confirm this idea, we analyzed the expression of Lk in individuals in which both Notch signalling was compromised and PCD was repressed (*mam*^{GA345}*, elav-Gal4>UAS-p35*). We observed extra ABLKs (χ =13.50±3.02*, Fig. R.13H, K and L), which confirmed the second hypothesis. Thus, we conclude that both postmitotic cells have the potential to differentiate into ABLKs, but are fated to die unless Notch signalling is activated. This only happens in the prospective ABLK, whereas the absence of N in the sibling leads to its apoptosis.

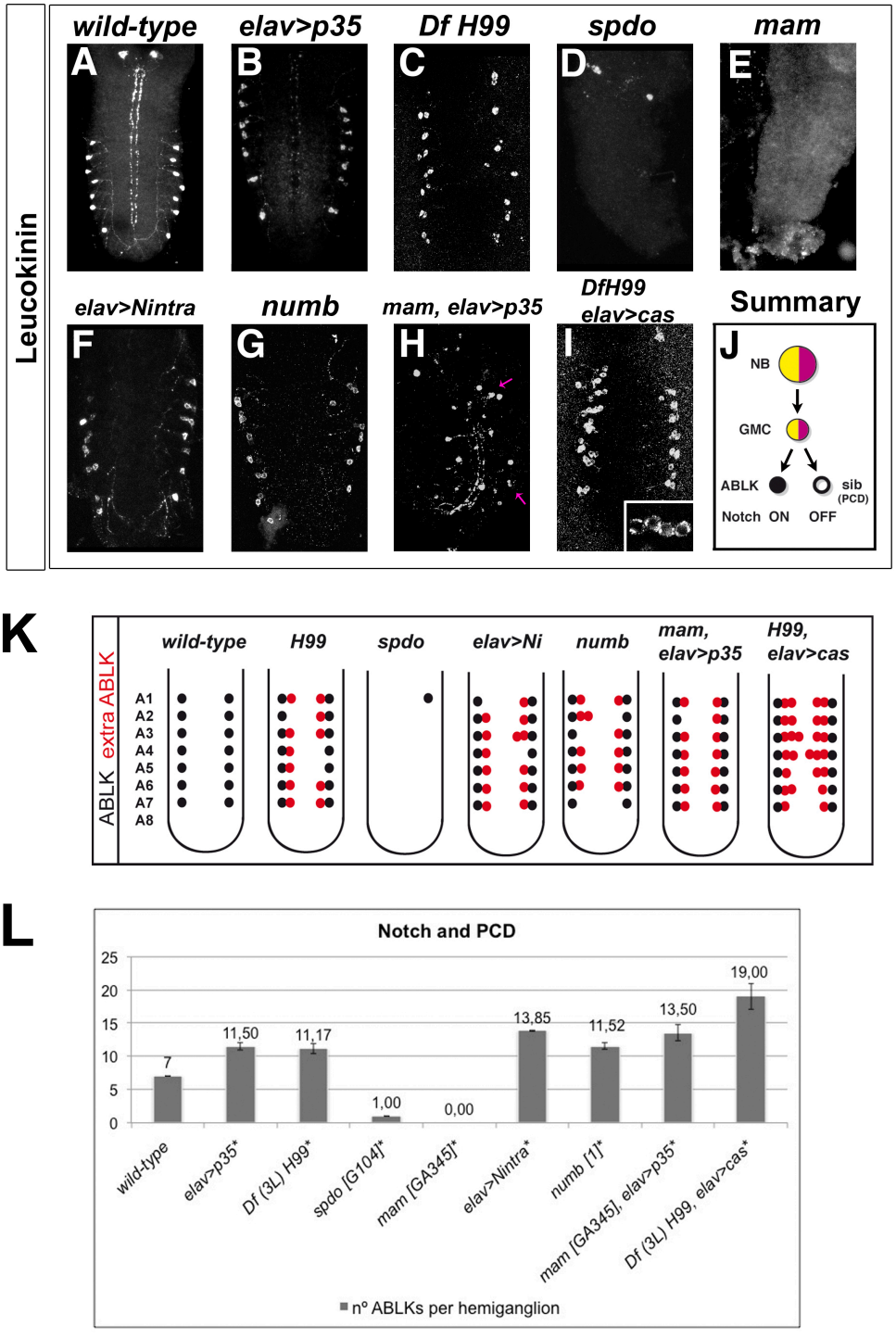


Fig. R.13. Requirement for Notch signalling in ABLK specification. (**A-I**) Expression of Lk in *wild-type* (A), *elav-Gal4>UAS-p35* (B), *Df(3L)H99* (C), *spdo*^{G104} (D), *mam*^{GA345} (E), *elav-Gal>UAS-Nintra* (F), *numb*^{1} (G), *mam*^{GA345}*, elav-Gal4>UAS-p35* (H) and *Df(3L)H99, elav-Gal4>UAS-cas* (I). ABLKs have a sibling cell that dies by apoptosis and is rescued when PCD is inhibited (B-C). ABLKs are Notch on and disappeared when this signalling pathway is inactivated (D-E) or they increased their number when Notch signalling is also activated in

the ABLK sibling cell (**F-G**). Both postmitotic neurons have the potential to differentiate as ABLKs, but are fated to die unless Notch signalling gets activated and rescues one of them (**H**). All the ventral nerve cords of A-I figures are of 18h AEL embryos. Arrows in H show two duplicated ABLKs. A magnification of a cluster of 4 ABLKs in shown in the inset of I. (**J**) Cartoon summarizing the above-mentioned results. (**K**) Cartoons summarizing the phenotypes observed. (**L**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type*.

So far, we have obtained duplications of ABLKs under two different conditions, *cas* misexpression (Fig. R.11B, J and K) and inhibition of PCD (Fig. R.13B-C, K and L). We assumed that the 2-3 ABLKs per hemisegment found upon *cas* misexpression were generated as a consequence of the prolongation of the ABLK window, whereas inhibition of apoptosis rescued the ABLK sibling cell. Therefore, when we misexpressed *cas* in *Df(3L)H99* mutant background (*Df(3L)H99, elav-Gal4 >UAS-cas*), we expected to obtain four times the number of ABLKs and, that is what we observed in some hemineuromeres (χ =19.00±3.92*, Fig. R.13I, K and L), which is a phenotype rarely detected upon *cas* misexpression alone.

6.6. Screening for genes involved in the specification of the ABLKs.

It is now assumed that neuronal fate specification is a multistep process that requires the combinatorial action of several regulators rather than the action of just one gene.

To find genes involved in the specification of ABLKs we followed two approaches. First, we tested genes that had already been reported to play a role or to be expressed in the development of the central nervous system of *Drosophila* (http://www.sdbonline.org/fly/aimorph/cns.htm). Second, we employed the deficiencies kit that Kai Zinn and colleagues compiled that covers around 50% of *Drosophila* genome. This kit is publicly available at Bloomington *Drosophila* Stock Center (Indiana University)(Wright et al., 2010), and provides a collection of deficiencies that produce homozygous embryos with relatively normal development to late stage 16, which was relevant to us since LK-expressing cells were first observed from 18h. AEL onwards.

We classified the studied genes in three different categories, the ones that did not produce phenotype, the ones in which the pattern of Lk was mildly altered and the ones in

which Lk expression was severely affected, either by decrease or increase in the number of ABLKs. Genes that did not produce phenotype or it was a mild one are shown in Table1 (Appendix I). Mutants in which the pattern of ABLKs was severely affected were *nab*, *klumpfuss* (*klu*), *jumeaux* (*jumu*), *teashirt* (*tsh*) and *knirps* (*kni*), so we focused on these genes.

Homozygotic mutants individuals for the strong allele nab^{R52}, or the transheterozygotic combination nab^{R52}/nab^{SH143} showed a reduced number of ABLKs (χ =0.00±0.00* and χ =0.10±0.32*, respectively). So it happened with the mutant alleles $klu^{212IR51C}$ (χ =0.00±0.00* and $klu^{212IR51C}/klu\text{-}lacZ$ χ =0.64±0.63*), *jumu* (χ =2.20±1.15*) and $tsh^{8}/Df(2L)Exel6049$ (χ =1.11±2.49*). In the case of kni^{6}/kni^{9}, we found duplications of ABLKs in all hemisegments (χ =13.00±0.00*). (Fig. R.14, R.16).

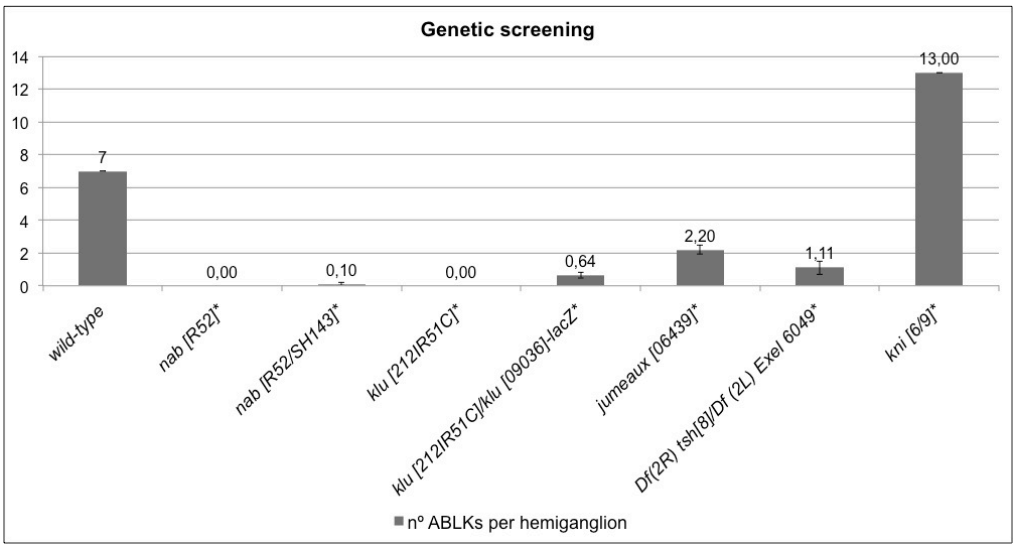


Fig. R.14. Genetic screening to find genes involved in ABLKs specification. Histogram showing the number of ABLKs in *wild-type*, nab^{R52}, $nab^{R2/SH143}$, $klu^{212IR51C}$, $klu^{212IR51C}/klu^{09036}$-*lacZ*, $jumu^{06439}$, $Df(2R)\ tsh^{8}/Df(2L)\ Exel6049$ and $kni^{6/9}$. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type*.

6.6.1. The genes *nab* and *klumpfuss* are required to specify the ABLK neuronal fate.

The Nab (NGFI-A-binding protein) protein is a nuclear cofactor without a DNA binding domain that acts by associating with other transcription factors (Russo et al., 1995; Svaren et al., 1996). In mammals, Nab proteins (Nab1 and 2) function as transcriptional co-regulators

that can repress (Svaren et al., 1998) or activate (Sevetson et al., 2000) gene expression by binding members of the Early growth factor (EGF) family of zinc-finger transcription factors (Clements et al., 2003).

In *Drosophila*, there is only one Nab protein and two EGF-like zinc-finger transcriptions factors, Stripe and Klu. Clements et al. (Clements et al., 2003), showed, by yeast two-hybrid assay, that *Drosophila* Nab bound to mammalian EGF proteins, but did not bind to the putative *Drosophila* EGF-like protein Klu. A similar experiment was not performed for Stripe since its expression pattern did not overlap the one of Nab, whereas so it did the one of Klu.

klu encodes a putative transcriptional regulator characterized by four C_2H_2 zinc-finger motifs in the C-terminus, distinguishing it from EGF mammalian proteins, which only have three, and making it more similar to the mammalian *Wilms tumor 1 (WT1)* gene (Klein and Campos-Ortega, 1997; Yang et al., 1997). Klu expression in the CNS starts at stage 10 and is present in 6 NBs per hemisegment; by late stage 11 its expression spreads to all of the 30 NBs, with the exceptions of NBs 2-3 and 6-4. It is also detected in some GMCs by this stage (Yang et al., 1997)(Fig. R.15A). Therefore, these data indicate that *klu* must be expressed in the ABLKs progenitor NB when it delaminates at late stage 11.

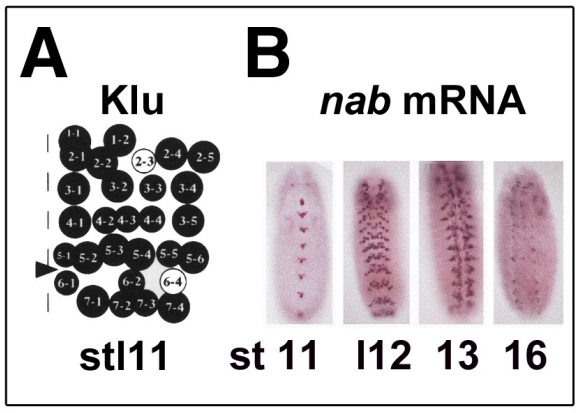


Fig. R.15. Pattern of expression of *klumpfuss* and *nab* in the development of the CNS of *Drosophila*. (**A**) Cartoon of one hemisegment showing the pattern of expression of Klu in a late stage 11 embryo. Anterior is up , dashed line indicates the midline and arrowhead marks the parasegmental groove. Extracted from (Yang et al., 1997). (**B**) Detection of *nab* mRNA in ventral views of embryos of stages 11, late 12, 13 and 16. Anterior is up. Modified from (Clements et al., 2003). (See main text for details).

On the other hand, we did not observe expression of Nab in NB5-5 at this stage (data not shown). *nab* expression in the CNS starts at stage 11, when its mRNA is only present in one NB per hemisegment. By stage 13, it is present in 10-12 NBs, and becomes undetectable from stage 16 till third instar larvae. At that point, *nab* expression is reactivated specifically in the

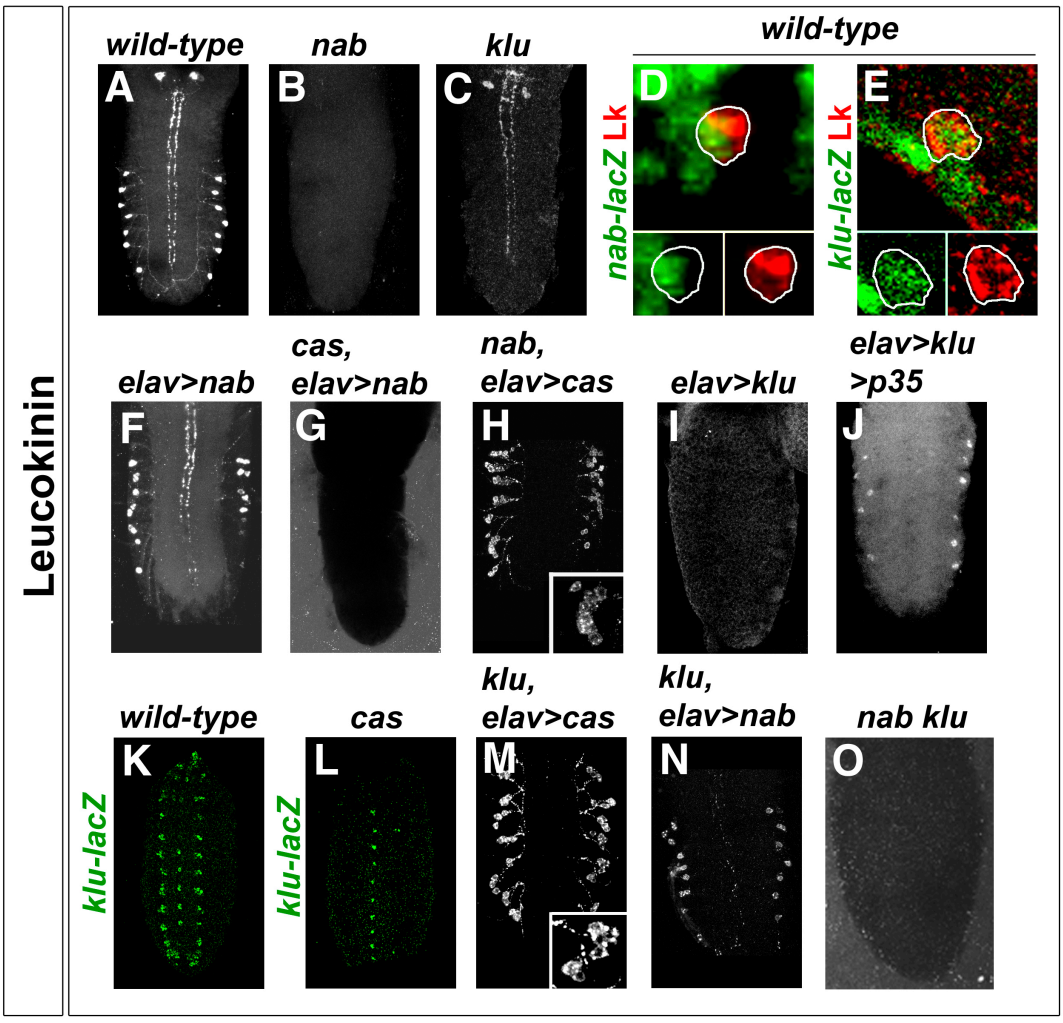
NBs of the thorax and cephalic lobes (Clements et al., 2003). Additionally, *nab* transcripts were absent in GMCs and neurons of the embryonic CNS. (Fig. R.15B).

Yet, we were able to detect expression of both genes, *klu* and *nab*, with their respective *lacZ* lines *(klu^{09036}-lacZ* and *nab^{SH143}-lacZ*) in ABLKs of 18h. AEL embryos (Fig. R.16D and E). It is important to note that we did not detect expression of Nab in ABLKs by immunostaining, suggesting that the presence of the *lac-Z* reporter in the postmitotic cell was due to perdurance.

We next wondered whether *nab* and *klu* could crossregulate each other at the level of transcription, so we checked for their expression in the respective mutant backgrounds and observed that expression of neither gene was affected in embryos mutant for the other (data not shown).

Misexpression of *nab* with the pan-neural driver (*elav-Gal4>UAS-nab*) produced duplications in some hemisegments as well as absence of ABLKs in others (χ =7.92 ± 1.45*, Fig. R.16F, P and Q). These extra cells expressed the temporal gene *cas* (data not shown), behaving as the bona fide ABLKs.

As presented before, ABLKs are born within a Cas temporal window, and all together, its loss-of-function, its misexpression phenotypes and its expression in the ABLKs indicates that this gene plays a pivotal role in the specification of ABLKs. It has been shown that Cas is able to activate *nab* expression. Clements et al., showed that there was a complete absence of both *nab* RNA and protein in *cas* mutants, it only remained the correspondent *nab* expression of the midline cells (Clements et al., 2003). Baumgardt et al., (Baumgardt et al., 2009) have recently described that Cas individually, and combinatorially with the zinc-finger Squeeze (Sqz) can also activate *nab* expression.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nab
klu
wild-type
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B
C
D
E
nab-lacZ Lk
klu-lacZ Lk
elav>nab
cas, elav>nab
nab, elav>cas
elav>klu
elav>klu >p35
F
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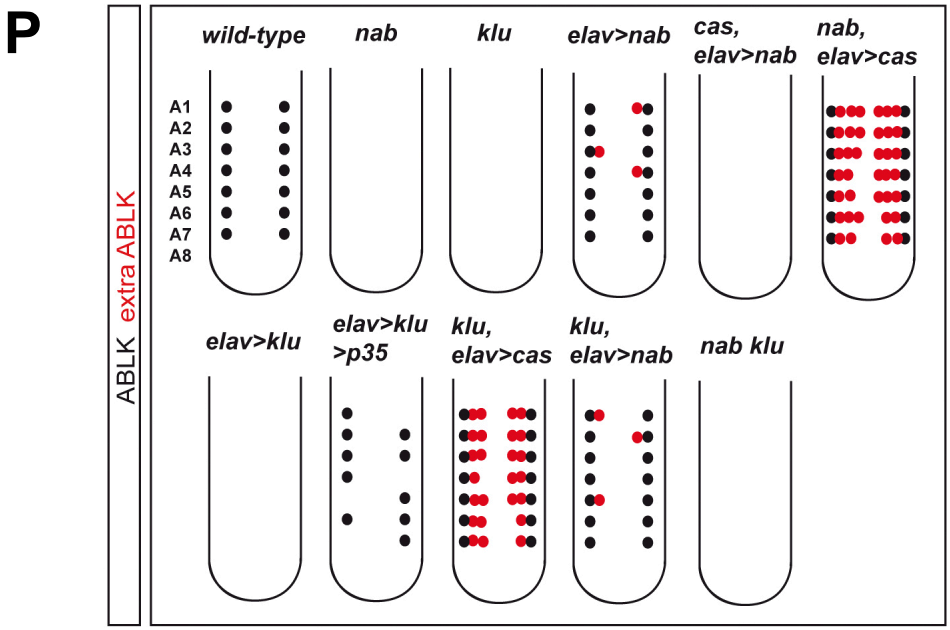
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wild-type
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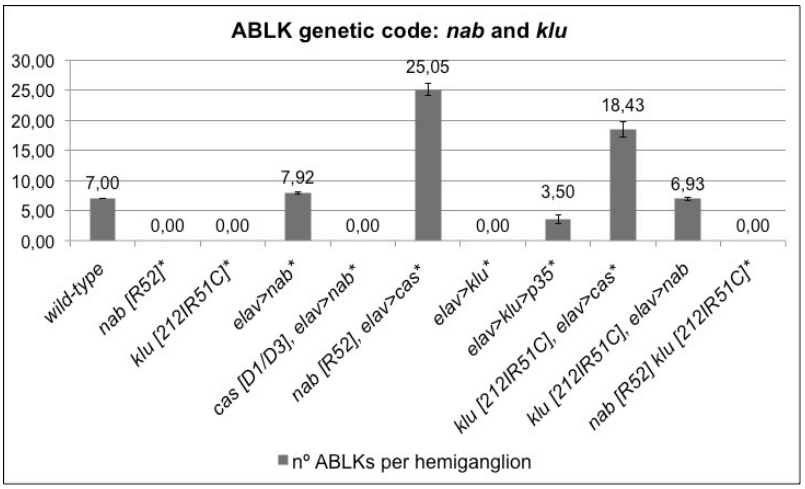


R.16. Nab and Klu are required for ABLK specification. (**A-C**) Expression of Lk in *wild-type* (A), nab^{R5} (B) and $klu^{212IR51C}$ (C) All ABLKs are lost in these two mutants . (**D,E**) Coexpression of Lk (red) and *nab-lacZ* (green; D) and *klu-lacZ* (green, E). A single ABLK is outlined in both cases, and separate channels are shown at the bottom of the figure. (**F-J**) Expression of Lk in *elav-Ga4>UAS-nab* (F), there are duplications of ABLKs in some hemisegments; $cas^{D1/D3}$, *elav-Gal4>UAS-nab* (G), all ABLKs are lost; nab^{R52}, *elav-Gal4>UAS-cas* (H), there are ectopic ABLKs in clusters, magnification of one hemisegment in the inset; *elav-Gal>UAS-klu* (I); ABLKs are lost; *elav-Ga4>UAS- klu UAS-p35* (J), there is a partial rescue in the pattern of expression of ABLKs. (**K,L**) Anti-β-galactosidase staining (green) in *klu-lacZ/+* (K) and *klu-lacZ* cas^{D1}/cas^{D3} (L), in stage 15 embryos. Expression of *klu-lacZ* disappears in *cas*. (**M-O**) Expression of Lk in $klu^{212IR51C}$, *elav-Gal4>UAS-cas* (M), there are ectopic ABLKs in clusters, magnification of one hemisegment in the inset; $klu^{212IR51C}$, *elav-Gal4>UAS-nab* (N), there is a oartial rescue in the pattern of expression of ABLKs; *and* nab^{R52} $klu^{212IR51C}$ (O), all ABLKs are lost. All ventral nerve cords from figures A-J and M-O are of 18h AEL embryos. (**P**) Cartoons summarizing the phenotypes observed. (**Q**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type*.

We wondered whether a similar mechanism was occurring during the specification of ABLKs. Therefore, if Cas was activating *nab* to specify the ABLKs, the solely misexpression of *nab* would be enough to rescue the absence of ABLKs that we obtained in a *cas* mutant background. However, when we performed the experiment, ($cas^{D1/D3}$, *elav-Gal4>UAS-nab*), we found no rescue (χ =0.00±0.00*, Fig. R.16G, P and Q), similar results were obtained using *wor-Gal4* as a driver (χ =0.00±0.00*, data not shown). Thus, it is clear that the role of Cas in ABLKs specification goes beyond than just activating *nab* expression.

We next overexpressed *cas* in a *nab* mutant background (nab^{R52}, *elav-Gal4>UAS-cas*) and, strikingly, found ectopic ABLKs (χ =25.05±4.61*, Fig. R.16H, P and Q), it is important to note

that this number was even higher that misexpressing *cas* alone. This result suggests that Cas, in addition to activating *nab* expression, acts either in parallel to, or downstream of Nab in ABLK specification (see Discussion).

On the other hand, misexpression of *klu* (*elav-Gal4>UAS-klu*) resulted in the complete absence of ABLKs. (χ =0.00±0.00*, Fig. R.16I, P and Q). It has been described a role of Klu in PCD during retinal development (Rusconi et al., 2004; Wildonger et al., 2005), so we attempted to rescue this phenotype by inhibiting apoptosis with overexpression of p35 (*elav-Gal4>UAS-klu UAS-p35).* We found that the pattern of ABLKs was partially restored (χ =3.50±1.87*, Fig. R.16J, P and Q), indicating that *klu* overexpression had a deleterious effect.

To determine whether the expression of *klu* depended on *cas*, we analyzed the expression of Klu in *cas* mutants (*klu-lacZ* $cas^{D1/D3}$). *klu* expression appeared normal in midline cells but failed to spread to NBs (Fig. R.16K and L). This result indicated that Cas activates *klu* expression. We did not test if this activation was enough to specify the ABLK fate code since the experiment required for that: ($cas^{D1/D3}$, *elav-Gal4>UAS-klu*), implied to misexpress *klu*, which we had seen to have a deleterious effect. On the other hand, and in light of the results obtained for *cas* and *nab*, we wondered if misexpression of *cas* was enough to rescue the absence of ABLKs seen in the loss of *klu* ($klu^{212IR51C}$, *elav-Gal4>UAS-cas)*. When we performed that experiment, we observed not only a rescue of the ABLK pattern, but also an increase in the number of cells, even more that with the single misexpression of Cas (χ =18.43±4.82*, Fig. R.16M, P and Q). This suggested that in the specification of ABLKs, Cas, in addition to activating *klu*, acts either in parallel to, or downstream of Klu. This result was reminiscent to the misexpression of Cas in a *nab* mutant background, both were able to rescue the pattern of ABLKs that was lost in the mutants, and on top of that, in both cases, this number of ABLKs was higher than in the only misexpression of Cas. A similar result was found in misexpression of Cas in a *grh* mutant background (Fig. R.11F, J and K).

We also observed that Nab overexpression in *klu* mutant embryos ($klu^{212IR51C}$, *elav-Gal4> UAS-nab*) rescued expression of ABLKs (χ =6.93 ±1.31, Fig. R.16N, P and Q). Furthermore, we studied the pattern of expression of Lk in double mutants for *nab* and *klu* ($nab^{R52}klu^{212IR51C}$), and as expected, found no rescue (χ =0.00 ±0.00*, Fig. R.16O, P and Q).

So far, we can conclude that ABLKs need Cas, Nab and Klu to be expressed in the NB that will generate them. About the hierarchies between them, Cas seems to act both upstream and downstream of both Nab and Klu. We next wondered about what was the specific function of Nab and Klu in ABLKs specification.

6.6.2. Nab and Klu are required to block the repressive activity of Squeeze on the ABLK fate.

As explained above, there is only one Nab protein in *Drosophila*, and since it lacks a DNA-binding domain, several candidates have been proposed to act as its partner. Terriente Félix et al.(Terriente Felix et al., 2007), showed, by pull-down assay, that Nab directly bound to the Zn-finger transcription factor Rotund (Rn) in the developing wing and transformed it from transcriptional activator to repressor, whereas it bound to Sqz in the CNS and limited the number of apterous-expressing neurons. Rn is not present in the CNS, but both Rn and Sqz are members of the Krüppel family of zinc-finger proteins (St Pierre et al., 2002) and share two highly homologous domains, the zinc-finger domain (90% identity) and a 32 amino acid C-terminal domain (over 80% identity), by which they bind to Nab (Terriente Felix et al., 2007). These data suggest that, in *Drosophila*, the partner of Nab would be Rn in the wing imaginal disc, but Sqz in the embryonic CNS development.

More recently, members of Stefan Thor´s lab have shed light on how this Nab/Sqz complex establishes a temporal subdivision of the Ap cluster neurons within the thoracic NB5-6 lineage (Baumgardt et al., 2009). This lineage generates around 20 cells, but the best-studied are the four neurons of the Ap cluster (Ap1-4) that are generated sequentially during the Cas/Grh temporal window. These cells comprise three different neuronal identities, the Ap1 and Ap4 neurons are neuropeptidergic and express the neuropeptides Nplp1 and FMRFamide (FMRFa), respectively, and the Ap2/3 interneurons, which are not peptidergic. The initial expression of Cas triggers the expression of Collier (Col), which acts to establish a "generic" Ap-neuron fate in all four Ap neurons. Later, the specific expression of the complex Nab/Sqz exclusively in Ap2/3 and Ap4 neurons downregulates the expression of *col* and allows for the acquisition of the different neuronal cell types within the Ap cluster (Fig. R17, extracted from (Kohwi and Doe, 2009)).

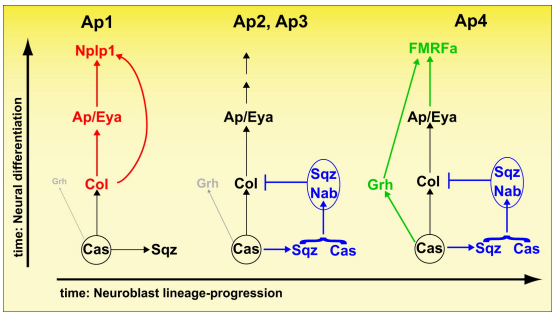


Fig. R.17. Generation of the Ap cluster by Cas feed-forward loops. Left column: a Cas->Col->Ap/Eya (red) results in the specification of Ap1/Nplp1. Middle: a Cas->Sqz feed-forward loop activates *nab* (blue). Sqz/Nab downregulate *col* expression in Ap2/3. Right column: a Cas->Grh (green) results in the specification of Ap4/FMRFa. Extracted from (Kohwi and Doe, 2009).

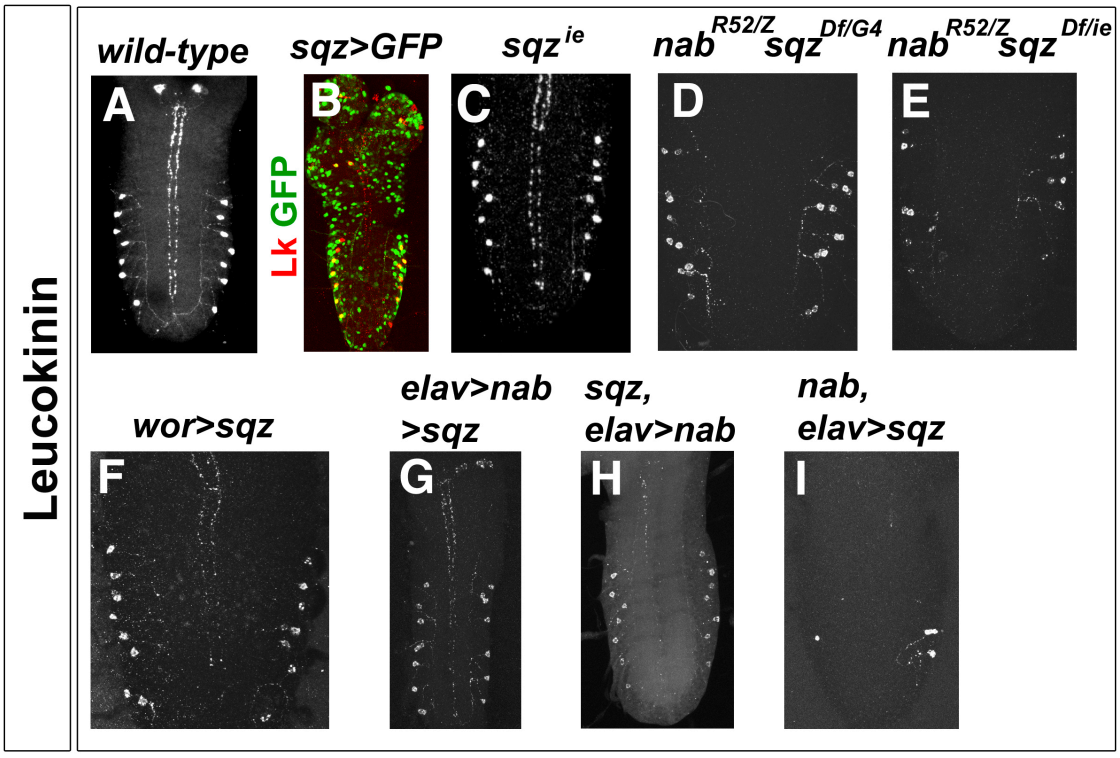
We wondered whether Nab and Sqz interacted in the ABLKs as it has been described before. It was reported that ABLKs expressed *sqz-lacZ* (*sqz*^{02102}) in third instar larvae (Herrero et al., 2007), and we confirmed it in 18h. AEL embryos (Fig. R.18B). As already mentioned, we did observe loss of ABLKs in *nab* embryos, however, LK expression was not altered in several *sqz* mutant backgrounds (*sqz*^{ie} and *sqz-Gal4*) (χ =7.00±0.00 Fig.R.18C, J and K). These results are in accordance with the ones already published by Herrero and collaborators, in which they determined that *sqz* was dispensable for the ABLKs at third instar larvae (Herrero et al., 2007). Nevertheless, this result has to be taken with caution as it has been reported that *sqz* has maternal effect (Perrimon et al., 1996).

We also analyzed the pattern of expression of ABLKs in *nab sqz* double mutants individuals. Strikingly, they showed an almost *wild-type* pattern. We tested several mutant combinations and obtained different numbers, ranging from 12 ABLKs in *nab*^{R52} *sqz*^{G4} (χ =12.00±2.12*), 9.35 in *nab*$^{R52/Z}$ *sqz* $^{Df/Gal4}$ (χ =9.35±3.28*), 7.75 in *nab*^{R52} *sqz*$^{Gal4/ie}$ (χ =7.75±2.05), 4.61 in nab$^{R52/Z}$ *sqz*$^{Df/ie}$ (χ =4.61±3.97*), and 0.92 ABLKs in *nab*^{R52} *sqz*^{ie} (χ =0.92±0.95*), per hemiganglion (Fig.R.18D-E, J and K). It is important to note that duplications of ABLKs appeared in all these cases, and also some hemisegments lacked ABLKs. This allelic series might be due to both the different nature of the *sqz* alleles studied and its maternal effect.

As shown above, misexpression of *nab* with *elav-Gal4* produced either duplications or loss of ABLKs in several hemisegments. We obtained a similar phenotype when misexpressing *sqz* with different drivers (*elav-Gal4*: χ =7.50±0.93 and *wor-Gal4*: χ =7.80±1.32*, Fig. R.18F, J and K). This phenotype was slightly stronger when both genes were co-misexpressed (*elav-Gal4>UAS-nab UAS-sqz,* χ =8.00±0.50*, Fig. R.18G, J and K).

So far we have detected expression of both *nab-lacZ* and *sqz-lacZ* in the ABLKs suggesting that they were present in the ABLKs-progenitor neuroblast and that their expression was inherited to the ABLK, at least for Nab, since no mRNA of Nab is present in GMCs and neurons (Clements et al., 2003). However, we cannot rule out the possibility that *sqz* is being actively transcribed in the progeny.

These results led us to propose a model in which Sqz would be repressing the fate of ABLK either directly via the repression of Lk expression, or indirectly, as it does in the specification of Ap4/FMRFa in the NB5-6T lineage. Nab would repress this role of Sqz by forming a complex with it, which will allow the specification of ABLKs. Consistent with this interpretation, loss of Sqz does not produce loss of ABLKs, given that *sqz* by itself is not required for their specification, it is need to be repressed. Conversely, loss of *nab*, prevents the inhibition of Sqz, whereas loss of both *nab* and *sqz* restores the *wild-type* phenotype. To confirm this hypothesis we performed cross-rescue experiments: $sqz^{ie/Gal4}$, *elav-Gal4>UAS-nab;* (χ =6.95 ±0.22, Fig. R.18H, J and K) and nab^{R52}, *elav-Gal4>UAS-sqz* (χ =1.33 ±1.92* Fig. R.18I, J and K). As expected, the overexpression of either of these genes does not disrupt the loss-of-funtion phenotype of the other.



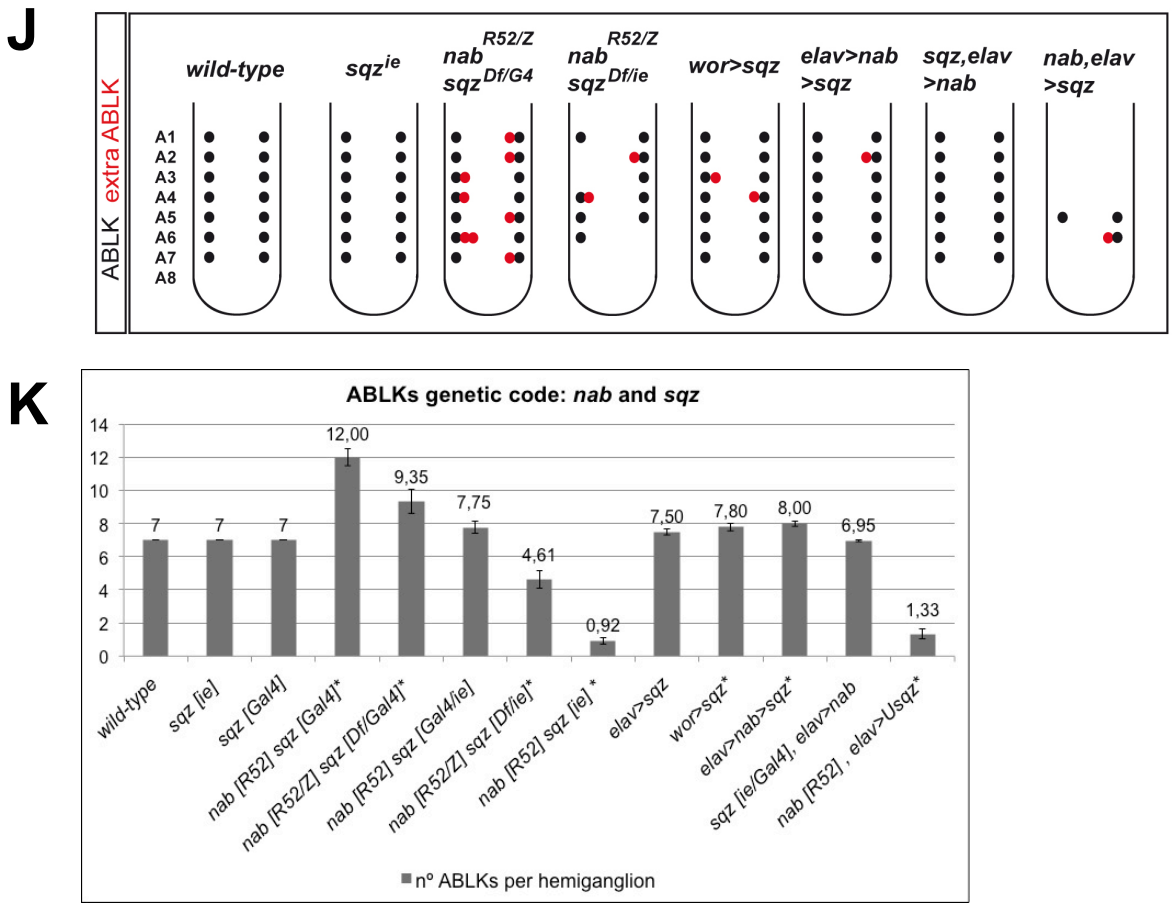


Fig. R.18. Nab is required to block the repressive activity of Sqz on the ABLK fate. (**A-I**) Expression of Lk in *wild-type*. (**B**) Coexpression of Lk (red) and GFP (green) in *sqz-Gal4>UAS-sqz*. (**C**) Expression of Lk in *sqz^{ie}*, the pattern of ABLKs is not affected. (**D**) Expression of LK in *nab^{R52}/nab^{SH143}-lacZ sqz$^{Df/G4:}$* there is a rescue of ABLKs, and duplications appeared in some hemisegments. (**E**) Expression of LK in *nab^{R52}/nab^{SH143}-lacZ sqz$^{Df/ie:}$* there is a also a rescue of the pattern of ABLKs, although they are missing in some hemisegments. (**F-G**) Expression of Lk in *elav-Gal4>UAS-sqz and* elav-Gal4>UAS-nab UAS-sqz*:* there are duplications of ABLKs in some hemineuromeres. (**H**) Expression of Lk in *sqz^{ie}, elav-Gal4>UAS-nab*: there is no phenotype. (**I**) Expression of LK in *nab^{R52}, elav-Gal4>UAS-sqz*: there is no rescue of ABLKs. The overexpression of either *nab* or *sqz* does not disrupt the loss-of-funtion phenotype of the other. All the ventral nerve cords of A-I figures are of 18h AEL embryos. (**J**) Cartoons summarizing the above-mentioned results. (**K**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type.*

We also observed that the pattern of ABLKs was rescued in *klu sqz* double mutants *klu^{212IR51C} sqz^{ie}* (χ =7.00 ±0.41, Fig. R.19B, D and E) *klu^{212IR51C} sqz^{Gal4}* (χ =7.31 ±0.60, Fig. R.19E), which suggested that the *klu* phenotype of loss of ABLKs was mediated by *sqz* action. Consistent with this model, misexpression of *sqz* did not alter *klu* phenotype (*klu^{212IR51C}*, *elav-*

Gal4; UAS-sqz χ =1.94 ±1.53*, Fig. R.19C, D and E). Therefore, *sqz* expression is not required, for ABLK specification. We do not know how Klu acts to block Sqz activity, if it does it at the level of transcription or at the protein level.

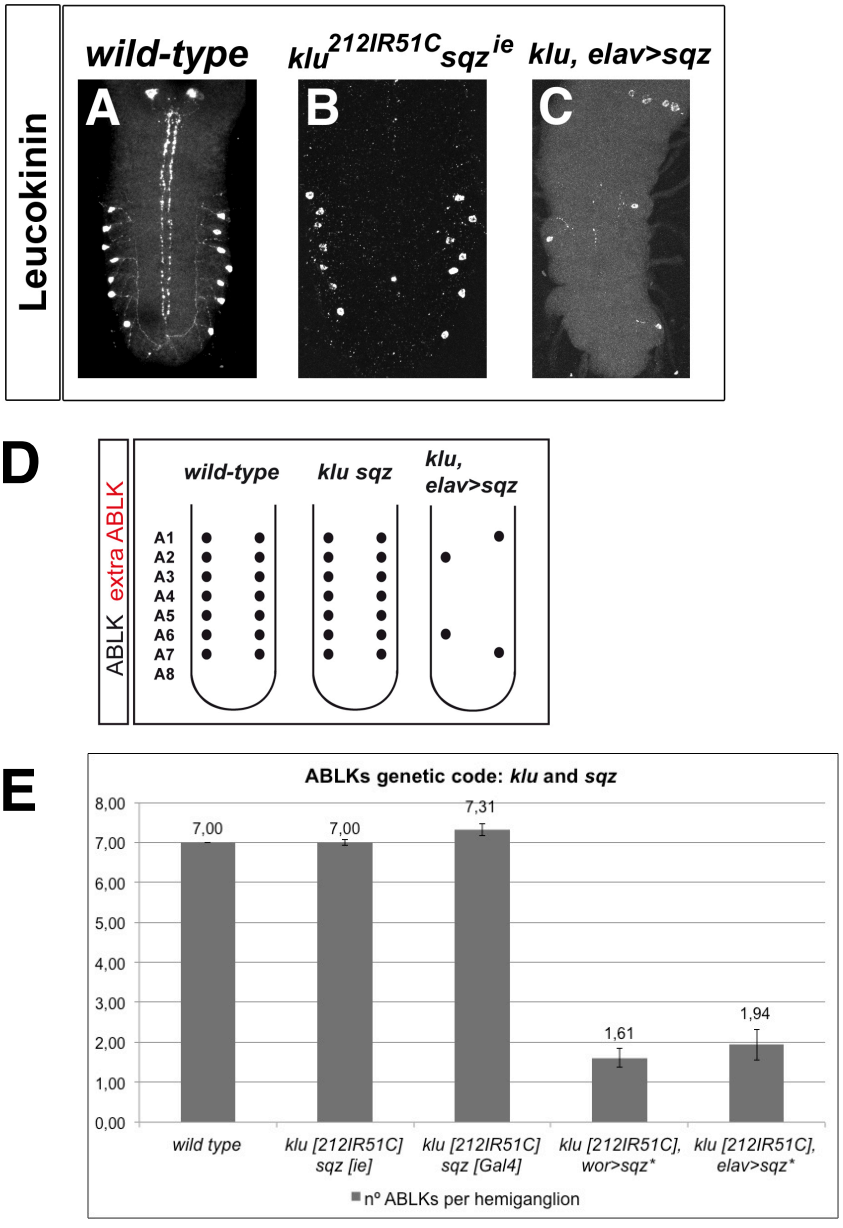


Fig. R.19. Klu is required to block the repressive activity of Sqz on the ABLK fate. (**A-C**) Expression of Lk in *wild-type* (A), *klu*^{212IR51C} *sqz*^{ie} (B) and *klu*^{212IR51C} and *elav-Gal4>UAS-sqz* (C) ventral nerve cords of 18h AEL embryos. The lack of ABLKs seen in *klu* mutants is rescued upon loss of *sqz* (B). (**D**) Cartoons summarizing the above mentioned results. (**E**) Histogram showing the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type.*

Together, the above-mentioned results of *nab*/*sqz*/*klu* and the ones we had concerning *cas* and *grh* suggest that Cas activates *sqz* (Baumgardt et al., 2009), *nab* (Clements et al., 2003), *klu* (this work) and *grh* (Baumgardt et al., 2009). It has been shown that *nab* can also be activated by Sqz in NB5-6T. However, due to the lack of molecular markers for NB5-5A lineage we cannot check for that possibility. According to the number of ABLKs, *nab* misexpression was not able to overcome the loss of *cas*. Therefore, we suggest that apart from activating nab, *cas* might also be downstream of *nab,* probably in the postmitotic cells. Additionally, misexpression of *cas* was enough to rescue the loss of ABLKs found in the mutant backgrounds for *nab* and *klu*. A model that integrates these findings will be presented in the Discussion section.

6.6.3. The gene *knirps* is involved in the specification of ABLKs.

We also observed duplications of ABLKs in *knirps* (*kni*^{6}/*kni*^{9}, χ =13.00±0.00*, Fig. R.20B, E and F). *kni* is a gap gene that has been described to function during the development of different tissues as embryonic head (Gonzalez-Gaitan et al., 1994), gut (Fuss et al., 2001), tracheal system (Chen et al., 1998) and wing disc (Lunde et al., 2003).

We then overexpressed *kni* with different drivers (*elav-Gal4*, and *wor-Gal4*) but we did not detect any phenotype (χ =6.93±0.26 and χ =7.00±0.00 respectively, Fig. R.20C, E and F). We also checked whether ABLKs expressed Kni at 18h. AEL embryos, but we did not find coexpression (Fig. R.20D). It is still remain to know if Kni is expressed at some point in the lineage of NB5-5; the proximity between the two ABLKs generated in *kni* mutants suggests that both cells belong to the same lineage, so Kni could be required to specify other neurons within NB5-5 lineage, and its loss would allow for the appearance of extra ABLKs.

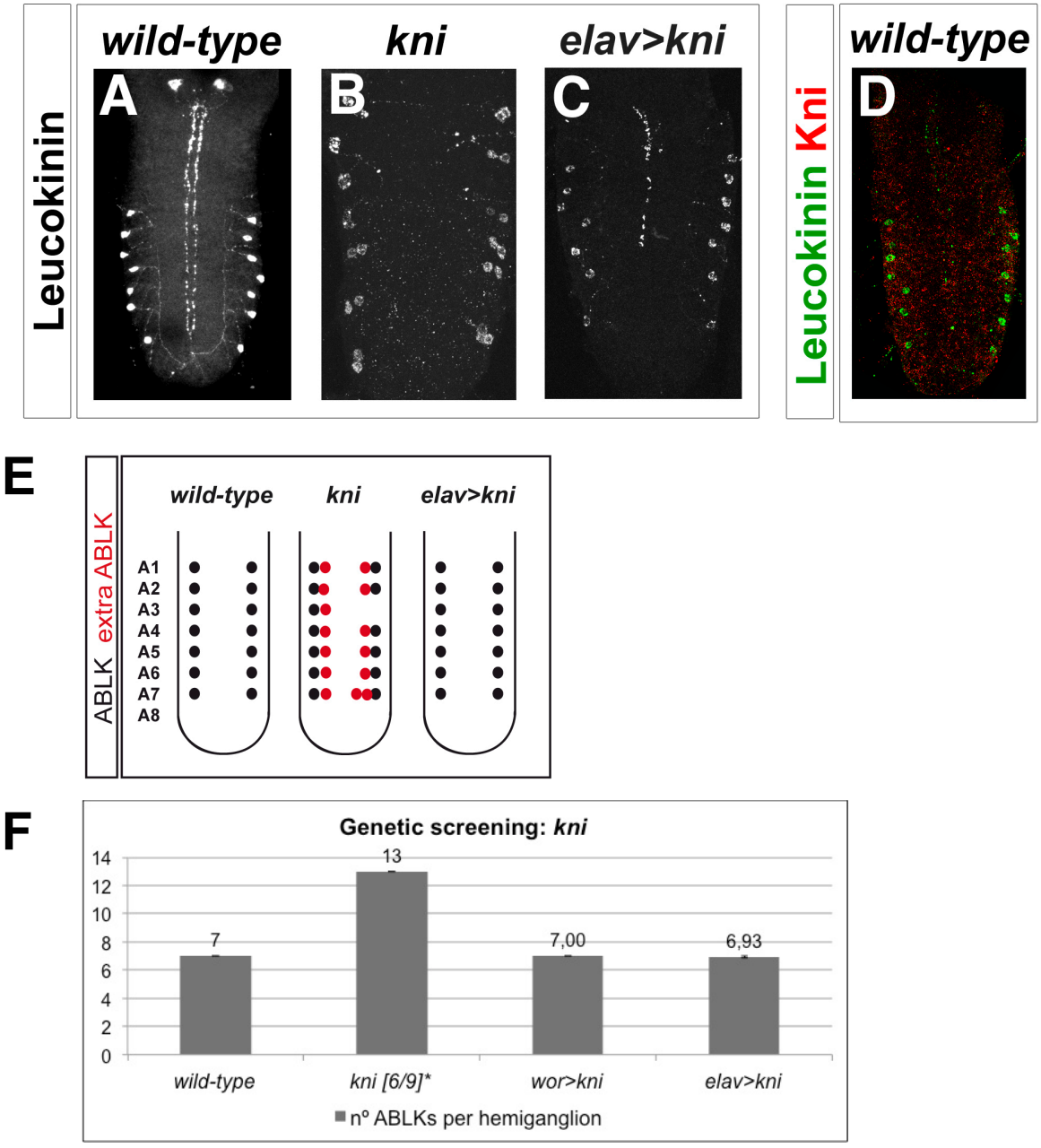


Fig. R.20. The gene *knirps* is involved in the specification of ABLKs. (**A-C**) Expression of Lk in *wild-type* (A), *kni*$^{6/9}$ (B) *elav-Gal4>UAS-kni* (C). Almost all ABLKs are duplicated in *kni* mutants, whereas its misexpression does not cause any phenotype (C). (**D**) Expression of Lk (green) and Kni (red). There is no expression of Kni at this stage. All the ventral nerve cords of A-D figures are of 18h AEL embryos. (**E**) Schematic showing the above-mentioned phenotypes. (**F**) Histogram depicting the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type.*

6.6.4 Summary of the Zinn deficiencies to identify new genes involved in ABLK specification.

We studied the pattern of ABLKs in a collection of 62 genetic deficiencies lines that uncovered most part of the second chromosome (Table 2 in Appendix II). In 16 of them we observed changes in the pattern of ABLKs that were statistically significant at 99% level of confidence (Fig. R.21 and R.22). For some of these deficiencies, we tried to identify which gene was producing the phenotype, for that we checked smaller overlapping deficiencies that uncovered the deleted region.

In the line *Df(2L)Exel6049* the pattern of ABLKs was severely reduced (χ =0.00±0.00*, Fig. R.21M and R.22). We identified the gene *teashirt (tsh)* as a possible candidate to account for this phenotype. Thus, we assessed ABLKs pattern in transheterozygous individuals for the mentioned deficiency and a mutant allele of *tsh (Df(2L)Exel6049/Df(2R)tsh^{8}),* and obtained a strong decrease in the number of ABLKs (χ =1.11±2.49*, Fig. R.23B and G). A similar result appeared in *Df(2R)tsh^{8}* homozygous embryos (χ =4.21±3.40*, Fig. R.23C, F and G).

Next, we misexpressed *tsh* with *elav-Gal4* and found ectopic ABLKs in some hemisegments (χ =7.56±0.89*, Fig. R.23D, F and G). However we did not find coexpression between ABLKs and Tsh in 18h. AEL embryos (Fig. R.23E). *tsh* encodes a zinc-finger transcription factor that is expressed in many tissues and is involved in several processes in the development of the fly; mainly, in the specification of the trunk versus the appendage in imaginal discs development (Fasano et al., 1991). Three putative orthologues genes have been identified in mouse that plays similar roles to *tsh* in flies (Manfroid et al., 2004). Moreover, *tsh* is decisive for the differentiation of a subset of secretory cells, stellate cells in *Drosophila* renal tubules, and among other genes, it controls the expression of the Leucokinin receptor (Denholm et al., 2013). However, this would not have to be directly involved in ABLKs specification. To our knowledge, no function has been described for *tsh* in the development of the CNS. Further experiments will be required to know its precise role in ABLK specification and its relationship with the other genes analysed above.

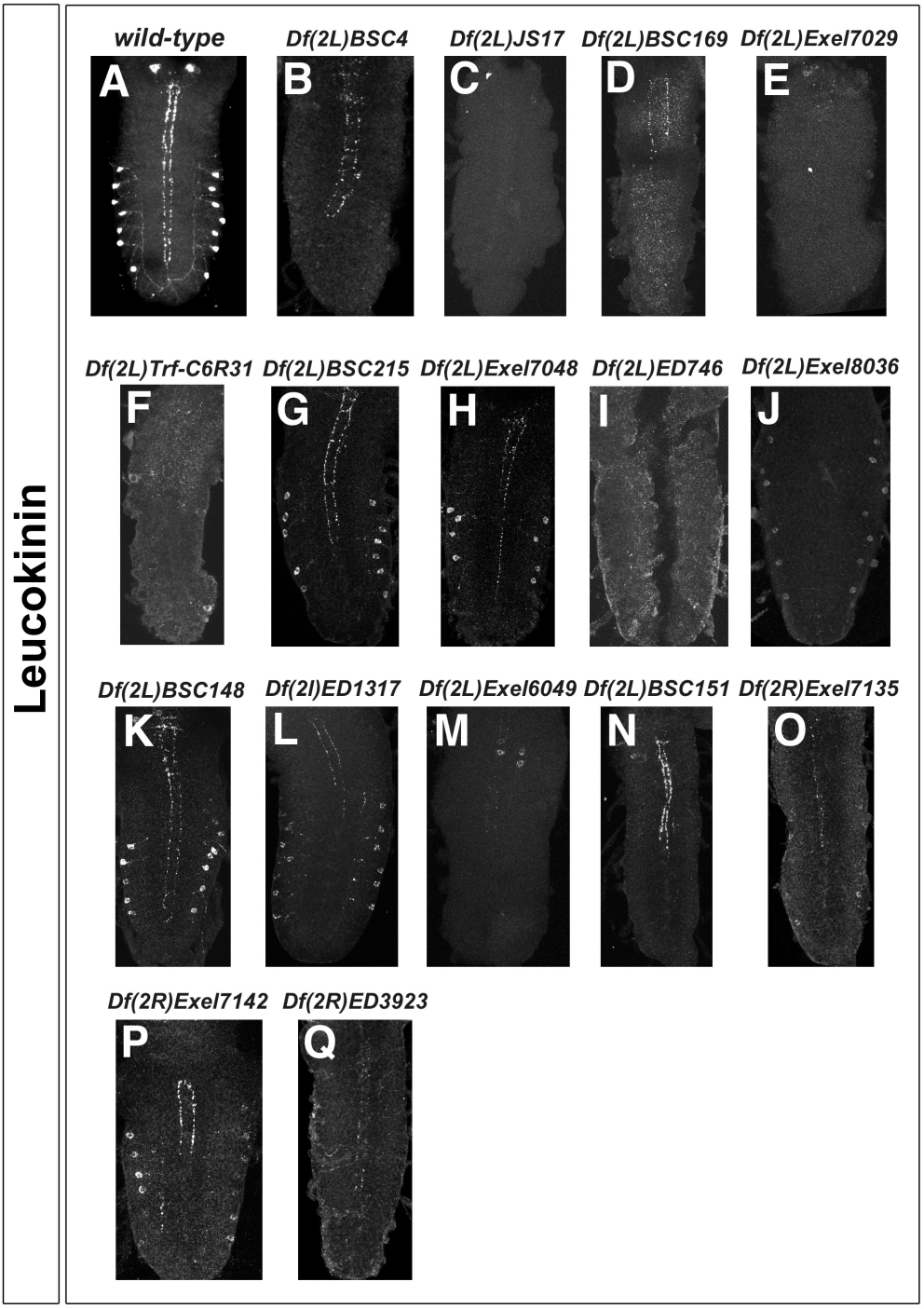


Fig. R.21. Zinn kit of genetic deficiencies with an altered pattern of expression of ABLKs. (**A-Q**) Expression of Lk in *wild-type* (A), *Df(2L)BS4* (B), *Df(2L)JS17* (C), *Df(2L)BSC169* (D), *Df(2L)Exel7029* (E), *Df(2L)Trf-C6R31* (F), *Df(2L)BSC215* (G), *Df(2L)Exel7048* (H), *Df(2L)ED746* (I), *Df(2L)Exel8036* (J), *Df(2L)BSC148* (K), *Df(2L)ED1317* (L), *Df(2L)Exel6049* (M), *Df(2L)BSC151* (N), *Df(2R)Exel7135* (O), *Df(2R)Exel7142* (P) and *Df(2R)ED3923* (Q). The number of ABLKs was strongly reduced in B-F, I, M-O and Q.

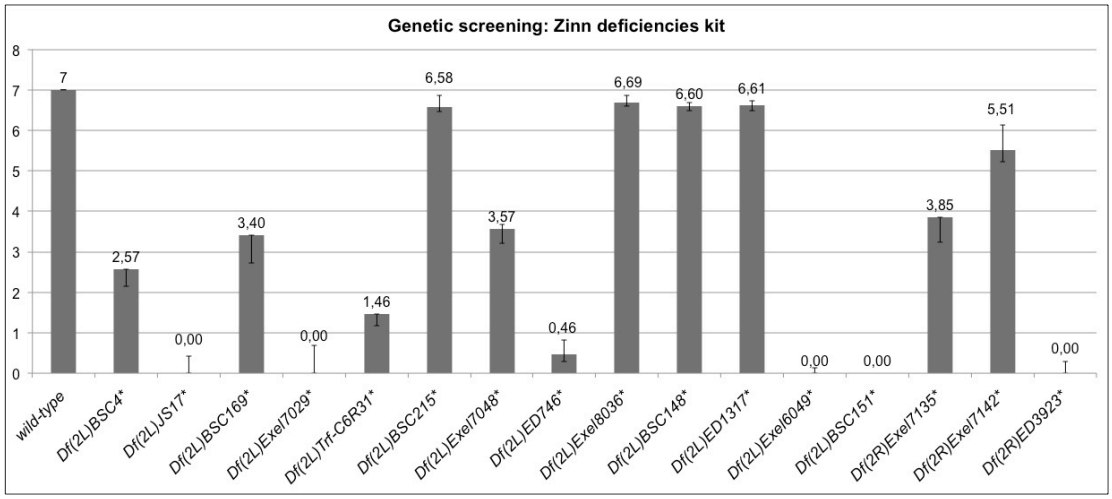
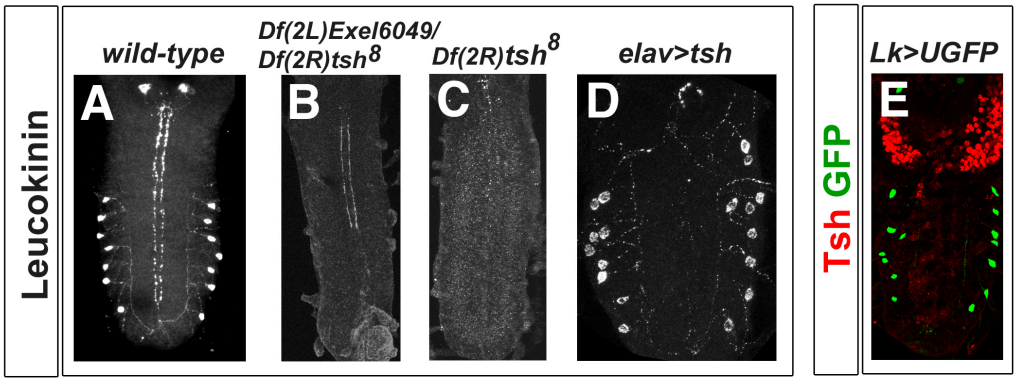
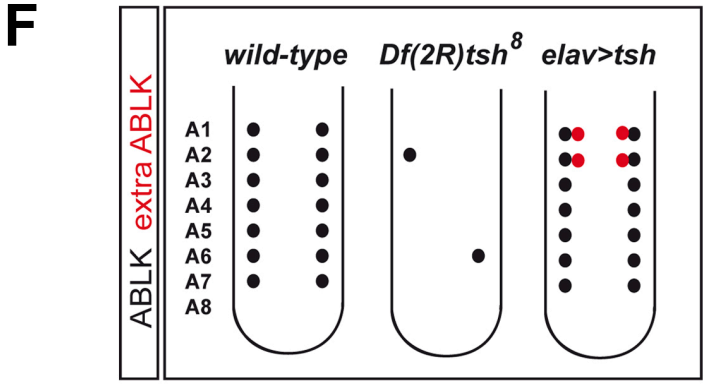


Fig. R.22. Zinn kit of genetic deficiencies with an altered pattern of expression of ABLKs. Histogram showing the average number of ABLKs per hemiganglion in the different deficiencies studied. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type.*





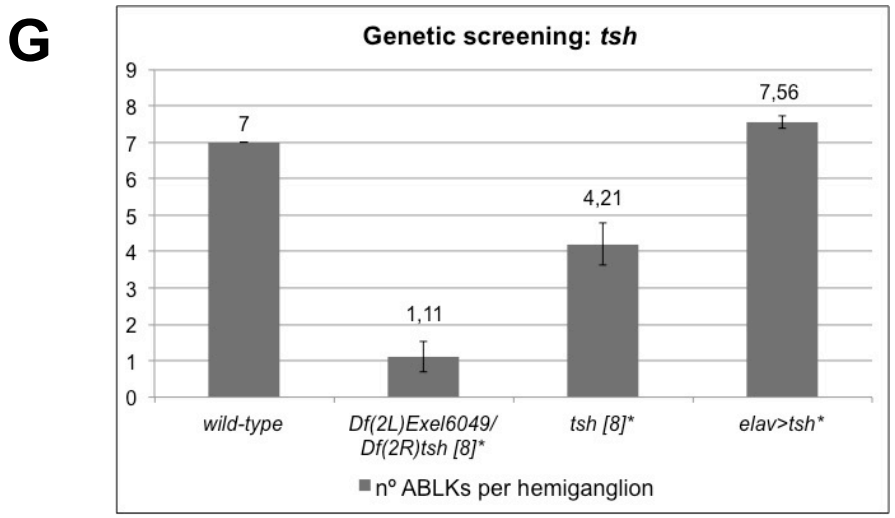
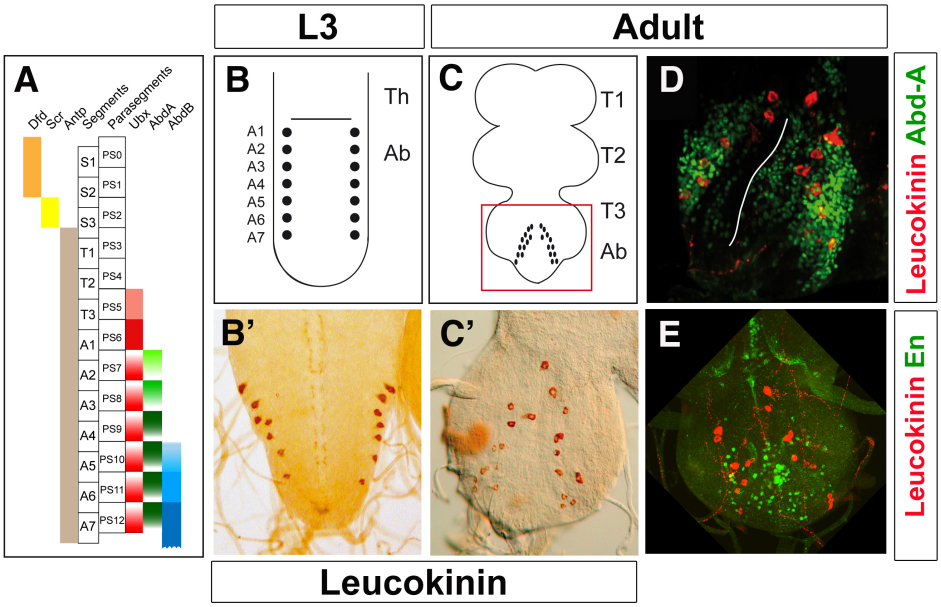


Fig. R.23. The gene *teashirt* is involved in the specification of ABLKs. (**A-D**) Expression of Lk in *wild-type* (A), *Df(2L)Exel6049/Df(2r)tsh*^{8} (B), *Df(2r)tsh*^{8} (C), *elav-Gal4>UAS-tsh* (D). The number of ABLKs is severely reduced in B and C. Misexpression of *tsh* generates duplications of ABLKs in some hemisegments (D). (**E**) Expression of GFP (green) and Tsh (red) in *Lk-Gal4>UAS-GFP*. All the ventral nerve cords of A-E figures are of 18h AEL embryos. (**F**) Schematic showing the above-mentioned phenotypes. (**G**) Histogram depicting the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type.*

The analysis of the other deficiencies that altered the pattern of ABLKs has not yielded, so far, the identification of other putative genes involved in ABLK specification.

6.7. Leucokinin expression in the ventral cord is initiated at two developmental stages.

As mentioned before the number of abdominal neurons that express Lk increased through development In the VNC of first instar larva there were 7 pairs of ABLKs, whereas 8 to 10 pairs were detected in adult flies (Herrero et al., 2003). Here, we found that the number of ABLKs started to increment by late pupal stages, reaching an average of 10 ABLKs per hemiganglion in 4-days-old adults (χ =10.75±0.17, Fig. R.24B-C and F).



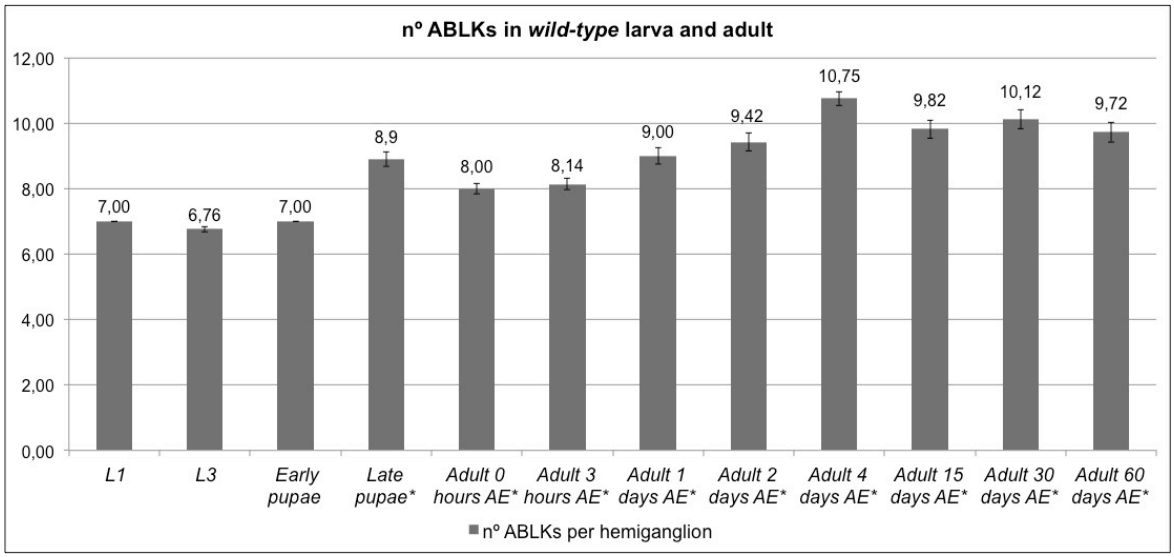


Fig. R.24. Pattern of ABLKs in the larval and adult CNS. (**A**) Schematic representation of the pattern of expression of Dfd (ochre), Scr (yellow), Antp (brown), Ubx (red), Abd-A (green)and Abd-B (blue) in the developing CNS. The different segments and parasegments are indicated (anterioir is upwards). (**B,C**) The pattern of ABLKs in the larval (B), which consists of a fixed number of 7 ABLKs per hemiganglion, and adult, which is formed by an average number of 10 ABLKs per hemiganglion, (C) CNS. Black circles represents ABLKs. Th, thorax; Ab, abdomen; S1-3, subesophagic segments; T1-3, thoracic segments; A1-7, abdominal segments. (**B'-C'**) Expression of Lk, detected using anti-Lk specific antibody, in the VNC of a third instar larva (B') and in the abdominal ganglion of an adult (C'). The red square of C indicates the area shown in C'. (**D,E**) Expression of Lk (red) and Abd-A (green, D) or En (green, E) in an abdominal ganglion of an adult fly. White lines depict the midline. (**F**) Histogram showing the number of ABLKs per hemiganglion throughout development, from first instar larvae to adults 60 AE (After eclosion). An asterisk next to the name of the genotype indicates that there were significant differences compared to first instar larvae *wild-type.*

As previously studied (de Haro et al., 2010), we did not detect any morphological differences between the ABLKs seen in larva and the new ABLKs of the adult. We tried to identify the segments from which these new ABLKs arose. For that we perfomed immunostaining with antibodies that either labelled a set of cells within a segment, as En (Fig. R.24D); or that marked a group of segments within the VNC, namely, Ubx and Abd-A. (Fig. R.24A, data not shown and Fig. R.24D, respectively). However, since the expression of these markers in the adult CNS did not reproduce the pattern seen in the larva, it was not possible to determine from which abdominal segments these new ABLKs came from.

6.8. ABLK neurons are generated during the embryonic and postembryonic neurogenesis.

VNC NBs undergo two phases of proliferation: the embryonic neurogenesis, which takes place in embryonic stages and makes up the larval nervous system, and the postembryonic or larval neurogenesis, which occurs from second instar larva onwards and contributes to most of the adult nervous system.

The ABLKs seen in first instar larvae were generated during the embryonic neurogenesis, but their onset of expression of Lk was delayed till 18h. AEL. We wondered whether the new ABLKs seen in adults were produced during this same embryonic neurogenesis but delayed their terminal differentiation (Lk expression) to adult stages, or, alternatively, whether they were generated during the postembryonic neurogenesis. To resolve this issue we traced the lineage of postembryonic NBs (pNBs). (Experiment 2 in Material and Methods). Using *wor-Gal4* as a NB driver, we labelled the whole lineage of pNBs with β-galactosidase from early second instar larva onwards (Fig. R.25A), and observed, in the adults, that only three or four cells were labelled with the tracer, and seven were not, per hemiganglion. (Fig. R.25C). This result indicated that the new ABLKs seen in adults were being generated during the postembryonic neurogenesis, and for that reason, we called them from then on postembryonic ABLKs (pABLKs). As a control, in the same lineage-tracing experiment, we checked the expression of the tracer β-galactosidase in third instar larva ganglia and confirmed that none of the 14 embryonic ABLKs (eABLKs) were labelled with the tracer. (Fig. R.25B).

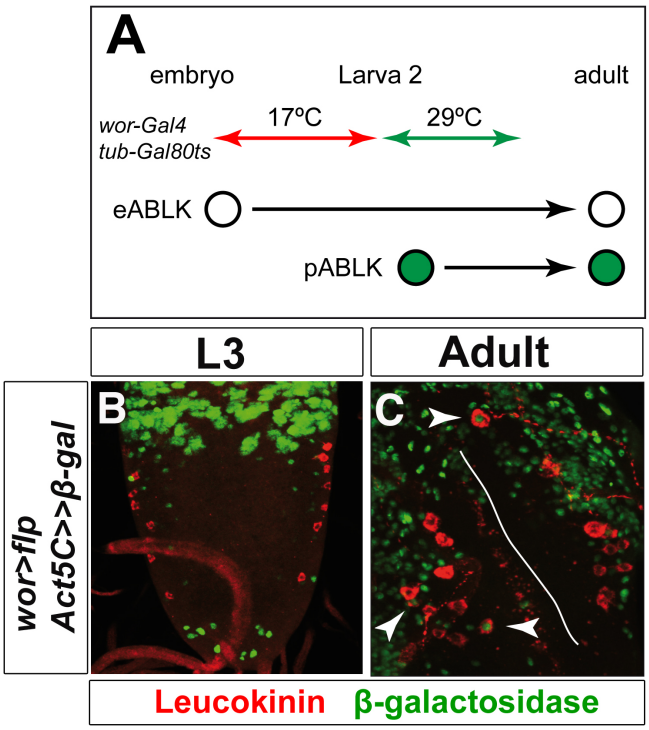


Fig. R.25. Adult ABLKs are generated during the larval neurogenesis. (**A**) The cell lineage experiment: *wor-Gal4>UAS-flp Act5C>stop> β-galactosidase tub-Gal80^{ts}* was temperature shifted from 17ºC to 29ºC in the early second instar larva; thus, only neurons generated during larval neurogenesis were labelled with β-galactosidase. (**B,C**) Result of the experiment depicted in A. Expression of Lk (red) and β-galactosidase (green) in third instar larva (B) and adult (C) VNC. White arrowheads indicate ABLKs that express β-galactosidase; the white line depicts the midline. Around 3-4 β-galactosidase labelled cells were found in adults (pABLKs) and 7 ABLKs were not labelled (eABLKs).

6.9. Embryonic and postembryonic ABLKs originate from the same progenitor neuroblast.

Since we found that eABLKs were generated by NB5-5, we wanted to know if this NB, later in development, also gave rise to the pABLKs. In central abdominal segments only three eNBs remain after embryonic neurogenesis, undergo quiescence and proliferate during larval stages. These pNBs are named according to their positions within the larval VNC: ventromedial (vm), ventrolateral (vl) and dorsolateral (dl) (Truman and Bate, 1988)(Fig. R.26A-B), but their embryonic identities have not been addressed so far.

First, among these three pNBs we wanted to determine which was the progenitor of pABLKs, for that, we sought for the coincidence in the expression of two molecular markers, *hkb* and *gsb*, in both the pNBs and the pABLKs. The markers expressed in NBs are usually maintained in their progeny. We observed that *hkb-lacZ* was expressed in the vlNB and dlNB in the VNC of second instar larva (Fig. R.26C and H), whereas *gsb-lacZ* had been reported to do so in the vmNB and vlNB (Almeida and Bray, 2005), (Fig. R.26F and H). On the other hand,

pABLKs were positive for both *hkb-lacZ* and *gsb-lacZ.* (Fig. R.26D, G and H). These findings strongly suggested that vlNB was the progenitor of pABLKs. To further confirm this conclusion, we traced the lineage of the pNBs (*nab-Gal4>UAS-flp Act5C>stop>β-galactosidase*). However, unlike clones induced in the embryo, in which the progeny usually forms a cluster (Prokop and Technau, 1991), cells belonging to these NB clone appeared scattered in the adult ganglia, making difficult to determine whether they belonged to a single clone or were the result of several recombination events. In addition, it was not possible to determine which pNB generated each clone in the adult.

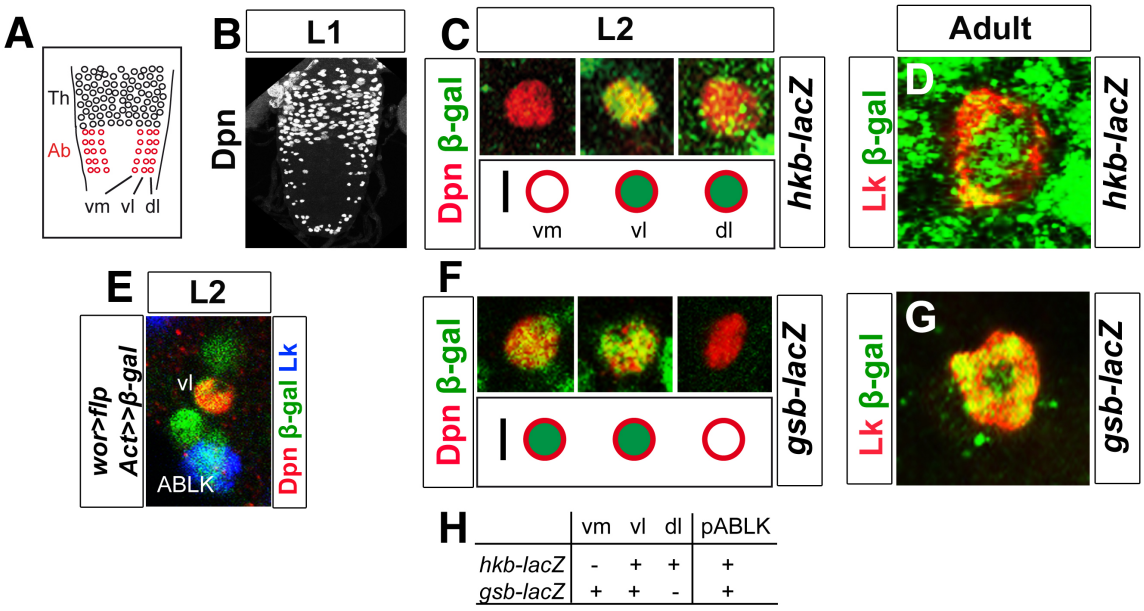


Fig. R.26. Embryonic and postembryonic ABLKs are generated by the same progenitor NB. (**A**) Schematic representation of neuroblasts (circles) in the larval VNC. Abdominal pNBs in the central abdomen (red): vm, ventromedial; vl,ventrolateral; dl, dorsolateral pNBs. (**B**) Dpn expression in the first instar larval VNC. (**C,F**) Expression of Dpn (red) and β-galactosidase (green) in the vm, vl and dl pNBs of *hkb-lacZ* (C) and *gsb-lacZ* (F). A schematic representation of the results is shown at the bottom of each image. Vertical bars depict the midline. (**D,G**) Expression of Lk (red) and β-galactosidase (green) in the ABLK of *hkb-lacZ* (D) and *gsb-lacZ* (G) adult ganglion. (**E**) Expression of Dpn (red), Lk (blue) and β-galactosidase (green) in clones (*wg-Gal4>UAS-flp Act5C>stop>β-galactosidase*) that label the embryonic progeny of the NB precursor of the vl pNB. The ABLK (blue) appears labelled, indicating that the vl pNB precursor in the progenitor of eABLKs. (H) Summary of the results shown in C,D,F,G. pABLKs share molecular markers with the vl pNB.

Next, we wanted to address if eNB5-5 was the same NB as vl pNB. For that, we traced the lineage of all eNBs of row 5 using a flip-in cassette and *wg-Gal4* to activate the Flippase

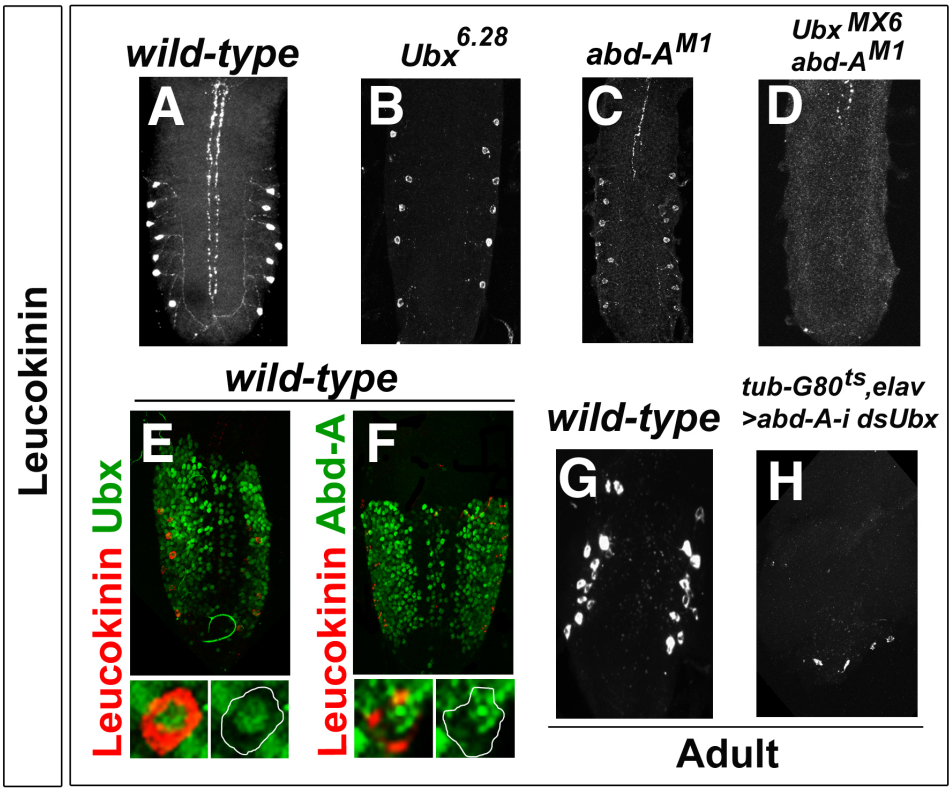
recombinase (*wg-Gal4>UAS-flp Act>stop>β-galactosidase*; see experiment 3 in Materials and Methods). We reasoned that if the same NB generated both embryonic and pABLKs, clones labelling the lineage of this NB should include the eABLK, indicating that the clone involved eNB5-5; a pNB, showing that eNB5-5 did not die after embryonic neurogenesis and resumed proliferation in larval stages; and the position of this pNB in the VNC must correspond to the vl pNB. This is indeed what we found (Fig. R.26E). Only clones that included the vlNB also included the eABLK. Thus, we infered that all ABLKs are generated from the same progenitor, namely the embryonic NB5-5 and the postembryonic vl.

6.10. Ubx and Abd-A are redundantly required to specify the ABLK fate

As it has been described that *Hox* homeotic genes participate in both vertebrates and invertebrates in the regulation of segment-specific nervous system development (Reviewed in (Carpenter, 2002; Estacio-Gomez and Diaz-Benjumea, 2013; Rogulja-Ortmann and Technau, 2008)), we wanted to know how they controlled the segment-specific appearance of ABLKs. To that end we analyzed the pattern of ABLKs in both loss-of-function and misexpression experiments of *Hox* genes.

We first analyzed individuals mutant for genes of the Bithorax complex and found that: in *Ultrabithorax* (*Ubx*) the ABLK of the segment A1 was lost ($Ubx^{6.28}$ χ =6.03 ±0.16*, Fig. R.27B, I and J), in *abdominal-A* (*abd-A*) the pattern was not altered ($abd\text{-}A^{M1}$ χ =7.00 ±0.00*, Fig. R.27C, I and J) and in *Ubx abd-A* all ABLKs were absent (Ubx^{MX6} $abd\text{-}A^{M1}$, χ =0.00 ±0.00*, Fig.R.27D, I and J; *Df(3R)109,* χ =0.00 ±0.00*, Fig. R.27J). On the other hand, in *Abdominal-B* (*Abd-B*) we observed an extra ABLK in segment A8 ($Abd\text{-}B^{M1}$ χ =7.98 ±0.15*, Fig. R.30B, I and J). The pattern of ABLKs was not affected in mutants for *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex comb reduced* (*Scr*) or *Antennapedia* (*Antp*) (Fig. R.27J).

Consistent with these results, we found that ABLKs expressed Ubx in segments A1-7, and Abd-A in segments A2-7 (Fig. R.27E and F). These data suggested that Ubx and Abd-A were redundantly required to specify the ABLK fate. Therefore, ABLKs were lost only when both genes were removed. In the case of the first abdominal segment, where only Ubx is expressed, its solely removal was enough to eliminate the corresponding ABLK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abd-AM1
Ubx MX6
abd-AM1
A
B
C
D
wild-type
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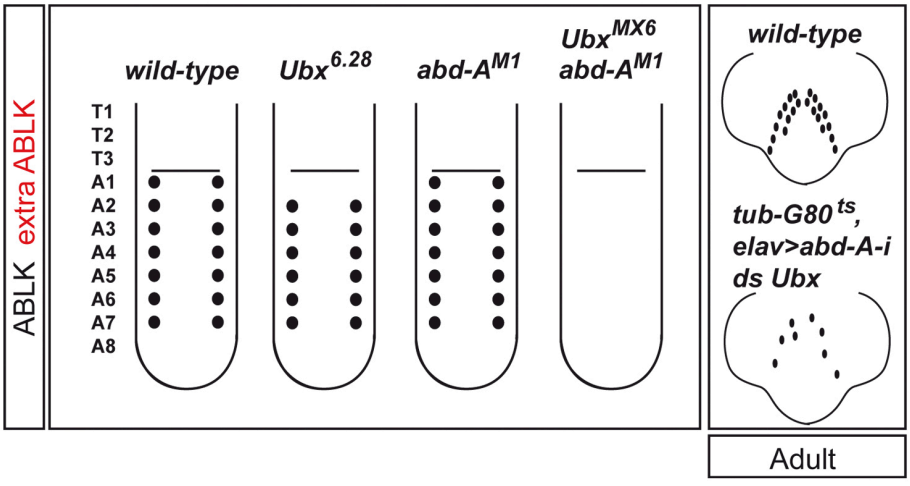
Leucokinin Ubx
Leucokinin Abd-A
Adult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UbxMX6
abd-AM1
T1
T2
T3
A1
A2
A3
A4
A5
A6
A7
A8
wild-type
tub-G80ts,
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ds Ubx
Adult

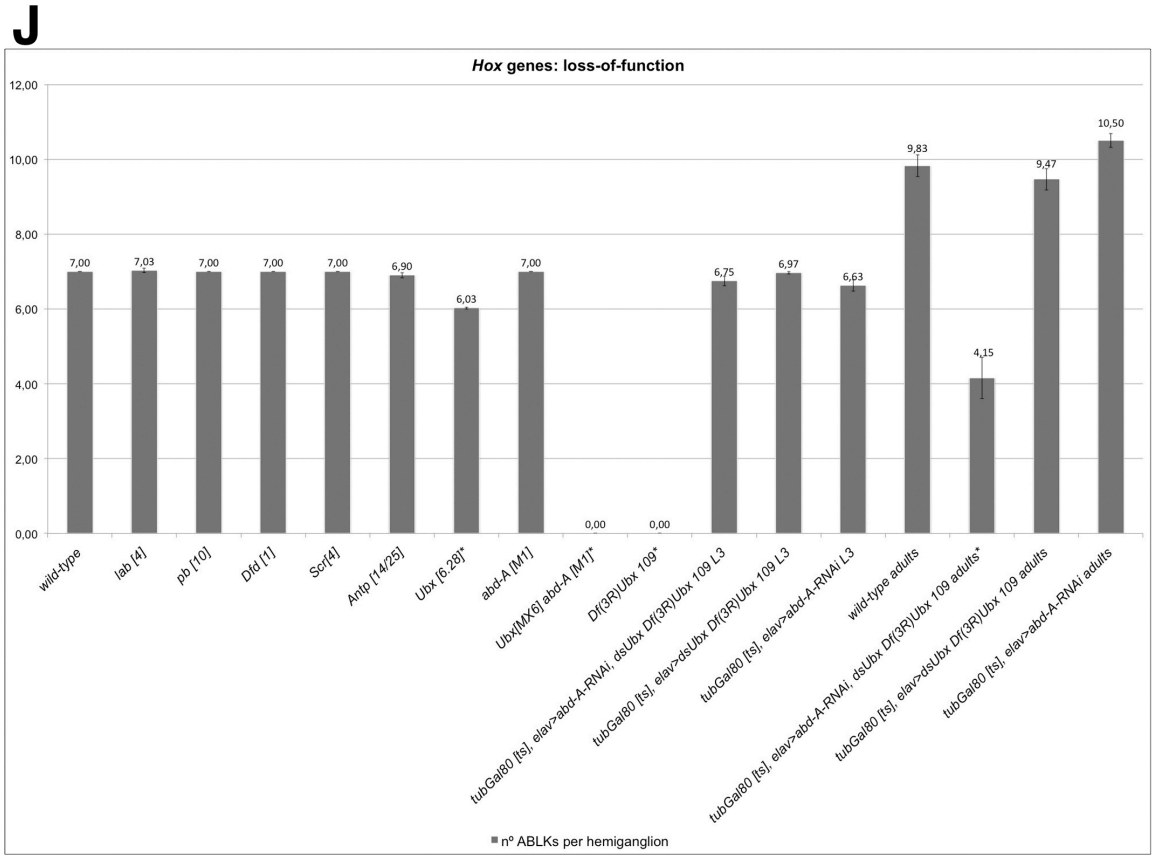


Fig. R.27. Ubx and Abd-A are required to specify the ABLKs. (**A-D**) Expression of Lk in *wild-type* (A), $Ubx^{6.28}$ (B), $abd\text{-}A^{M1}$ (C) and Ubx^{MX6} $abd\text{-}A^{M1}$ (D) VNCs of 18h AEL embryos. The correspondant ABLK of A1 is lost in *Ubx* (B) and all ABLKs are missing in *Ubx abd-A* double mutants (D). (**E,F**) Coexpression of Lk (red) and Ubx (green, E) and Abd-A (green, F) in *wild-type* 18h AEL embryos. Ubx coexpresses with ABLKs in all abdominal segments (A1-7) and Abd-A from A2-7. (**G,H**) Expression of LK in *wild-type* (G) and *tub-Gal80^{ts}, elav-Gal4>UAS-abd-A-RNAi UAS-ds Ubx* VNCs of adults. Most of ABLKs are lost in adults when both *Ubx* and *abd-A* are knocked down from first instar larvae. (**I**) Schematic showing the phenotypes observed. (**J**) Histogram depicting the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type.*

As we found expression of Ubx and Abd-A in the ABLKs at 18h. AEL, we wondered whether they were also required for the maintenance of Lk expression. To test this, we knocked down the expression of both *Ubx* and *abd-A* from first instar larva and stained for LK expression in either late third instar larva or adult fly (*tub-Gal80^{ts,} elav-Gal4>UAS-abd-A-RNAi UAS-dsUbx*). (For details see experiment 4 in Materials and Methods). At third instar larva we observed that most of ABLKs were still present (χ =6.75 ±0.77, Fig. R.27J), whereas many of them disappeared in adults (Fig. χ =4.15 ±2.80*, Fig. R.27H, I and J). When we knocked down these

genes individually, we did not detect any changes in ABLKs expression, nor in third instar larva (*tub-Gal80^{ts}, elav-Gal4>UAS-dsUbx:* χ =6.97 ±0.19 and *tub-Gal80^{ts}, elav-Gal4>UAS-abdA-RNAi:* χ =6.63 ±0.62, Fig. R.27J), nor in adult (*tub-Gal80^{ts}, elav-Gal4>UAS-dsUbx:* χ =9.47 ±1.13 and *tub-Gal80^{ts}, elav-Gal4>UAS-abdA-RNAi:* χ =10.50 ±0.73, Fig. R.27J). These observations indicate that Ubx and Abd-A are also redundantly required in ABLKs to sustain the expression of Lk. Since the removal of both genes turned out to be essential for the maintenance of LK expression, we expected the corresponding ABLK from A1 to disappear in adults when only Ubx was knocked down, similarly to what happens in *Ubx* in 18h. AEL embryos. However, the lack of this phenotype can be explained by the inefficiency of the *UAS-dsUbx* line to completely remove Ubx expression, as it suggests its presence in immunostaning.

Next, we ectopically expressed *Ubx* (*elav-Gal4>UAS-Ubx*) and found one extra ABLK in segments T1-3 (χ =9.80 ±0.41*, Fig. R.28B, F and G). On the other hand, ectopic expression of *abd-A* (*elav-Gal4>UAS-abd-A*) generated clusters of two to four ABLKs, mostly in segments T1-A4 (χ =16.87±4.51*, Fig. R.28C, F and G). These extra ABLKs were identical to the bona fide ones in terms of morphology and axonal projections (data not shown).

We wondered about the origin of these extra ABLKs and considered three different scenarios: first, these ectopic ABLKs were the sibling cells of the bona fide ones that usually die by apoptosis (Benito-Sipos et al., 2010) but that, in this experiment, were rescued. Second, as Abd-A was required for the specification of the ABLKs, its misexpression could be enough to trigger ABLK expression or to change the specification of some other neuron of the lineage. Third, these extra ABLKs were the pABLKs that, in *wild-type* development, are born during the larval neurogenesis, but upon *abd-A* misexpression, NB entry into quiescence is prevented and, consequently, NB5-5 executes its full embryonic and larval program without interruption. To distinguish between these three possibilities we performed the following experiments: First, we misexpressed both *abd-A* and *p35 (elav-Gal4>UAS-p35>UAS-abd-A)*. If the ectopic ABLKs seen upon *abd-A* misexpression corresponded to the rescued sibling cells, misexpression of *abdA* together with *p35* should generate the same number of ABLKs; if otherwise we obtained an increase in the number of ABLKs, we could conclude that those ectopic ABLKs that appeared in *abd-A* misexpression were not the ABLK sibling cells. This was indeed what we found (χ =21.10±4.60*, Fig. R.28D, F and G) thus, we ruled out the first explanation.

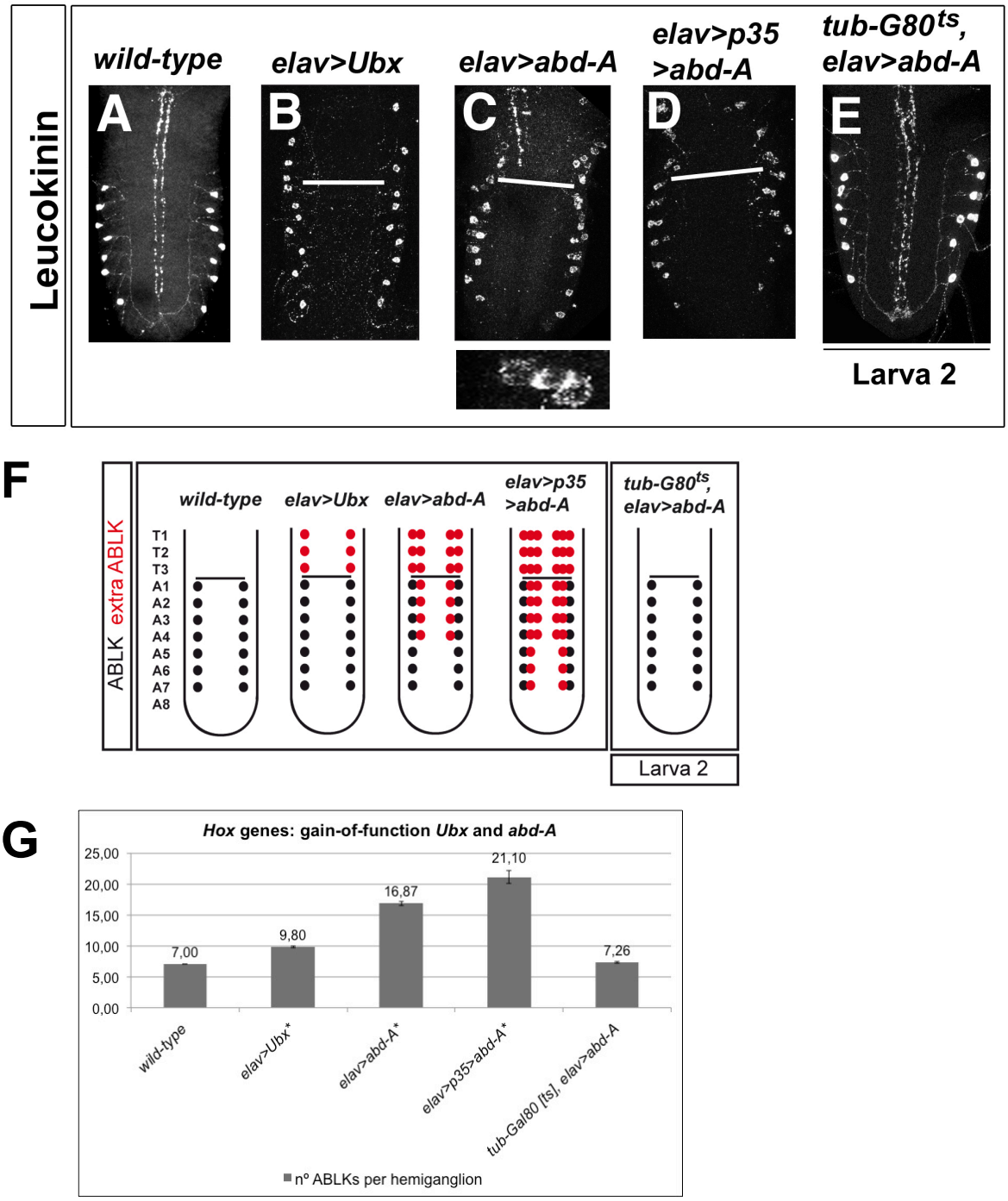


Fig. R.28. Ubx and Abd-A are redundantly required to specify the ABLKs. (**A-D**) Expression of Lk in *wild-type* (A), *elav-Gal4>UAS-Ubx* (B), *elav-Gal4>UAS-abd-A* (C) and *elav-Gal4>UAS-p35 UAS-abd-A* (D) in VNCs of 18h AEL embryos. Horizontal bars depict the boundary between the thorax and abdomen. One ectopic ABLK appeared in each of the thoracic segments when misexpressing *Ubx* (B). Misexpression of *abd-A* generated clusters of 2-4 ectopic ABLKs mostly from T1 to A4 (C). Misexpression of both *abd-A* and *p35* increased the number of ectopic ABLKs seen upon *abd-A* misexpression, suggesting that the ectopic ABLKs seen were not the ABLKs sibling cells. (**E**) Expression of Lk in *tub-Gal80^{ts}, elav-*

Gal4>UAS-abd-A, temperature shifted to 29ºC at first instar larvae, in VNC of second instar larva. This misexpression of *abd-A* is not enough to significantly generate ectopic ABLKs. Thus, we propose that entry into quiescence is prevented upon abd-A misexpression, and the ectopic ABLKs seen in 18h AEL embryos, are the pABLKs. (**F**) Schematic showing the phenotypes observed. (**G**) Histogram depicting the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type.*

Next, we misexpressed *abd-A* from first instar larva and checked ABLK pattern at second instar larva (see experiment 5 in Materials and Methods), to see if its addition was sufficient to transform other neurons towards ABLKs (*tub-Gal80^{ts}, elav-Gal4>UAS-abd-A).* We did not observe any phenotype (χ =7.26±0.71, Fig. R.28E, F and G), suggesting that, although Abd-A is required to activate the ABLK fate, its expression in postmitotic cells in the larva is not enough to produce new ABLKs.

Nevertheless, the two above-mentioned scenarios are not mutually exclusive; Abd-A could be required during embryonic development to specify the eABLKs, and, at the same time, the pABLKs, which are normally generated in larval neurogenesis and detected in the adult, could be generated in the embryo upon *abd-A* misexpression. This would imply that if the entry into quiescence was prevented, the developmental program of NB5-5, which is normally divided into two phases, embryonic and larval, would develop without interruption.

An additional observation that favours this interpretation is the different phenotypes observed upon *abd-A* and *p35* misexpression. In the first one, the ectopic cells of the abdomen were mostly found from the first thoracic to the fourth abdominal segment, and sometimes more than 2 cells were seen. However, misexpression of *p35* produced duplications in all abdominal segments, without favouring ones in detriment of others.

A similar situation has been described in the thoracic NB3-3, which normally enters quiescence by the end of the embryonic period, late stage 14 to stage 15, and which, upon misexpression of *abd-A* or loss of *Antennapedia*, it does not enter quiescence and keeps dividing, generating, in the embryonic neurogenesis, neurons that are normally detected during the larval phases (Tsuji et al., 2008). In that study they were able to distinguish between the neurons produced during the embryonic and postembryonic periods by labelling with either Krüppel or Lin29, which are expressed in early or late-born neurons, respectively.

To check if Lin29 was differentially expressed among the ectopic ABLKs, we stained both *wild-type* ganglia and ganglia in which *abd-A* was misexpressed with it. The idea was to distinguish between the embryonic, and the postembryonic ABLKs that, we assume, appear when *abdA* is misexpressed. We found that Lin29 was expressed in all the ABLKs in *wild-type* ganglia (Fig. R.29A), whereas not all of the ectopic ABLKs that appeared upon *abd-A* misexpression expressed this marker (Fig. R.29D). Moreover, these cells did not exactly correspond to the number of the ectopic ones. Therefore, in our case, we cannot use Lin29 to distinguish between these two temporally differentiated populations of neurons.

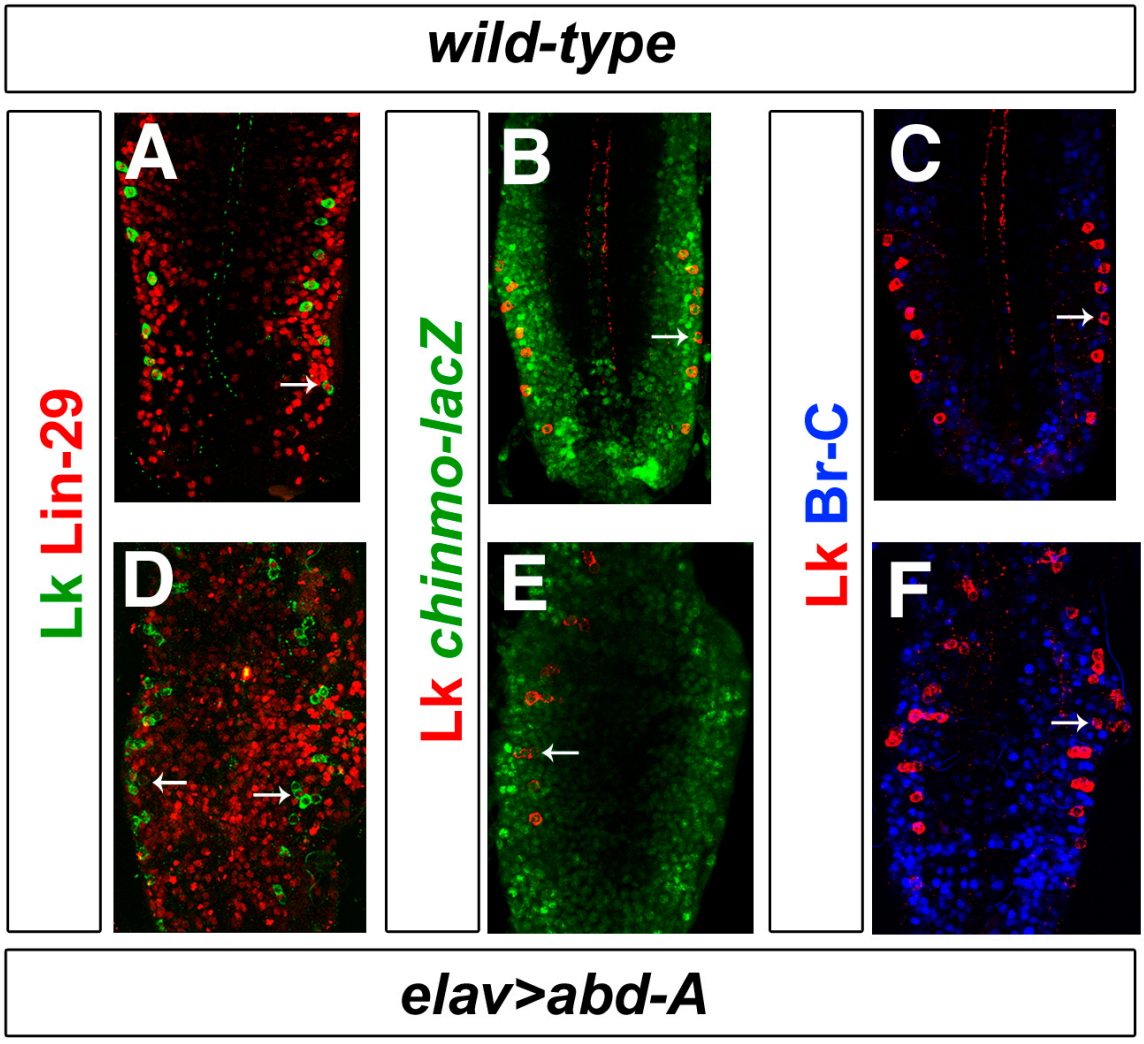


Fig. R.29. Colocalizations between ABLKs and temporal molecular markers. (**A,D**) Expression of Lk (green) and Lin-29 (red) in *wild-type* (A) and in *elav-Gal4>UAS-abd-A* (D). All ABLKs coexpress Lin-29 in *wild-type* embryos. See white arrow in A. (D) Not all the ABLKs

seen upon *abd-A* misexpression coexpress Lin-29, and this number does not correspond to either the bona fide ABLKs nor the extra ABLKs. See left white arrow; out of 3 extra ABLKs only 2 are labelled with Lin29. Also in (D), the right white arrow indicates a cluster of 4 ectopic ABLKs in which only one is labelled with Lin29. (**B,E**) Expression of Lk (red) and β-galactosidase (green) in *chinmo-lacZ* (B) and in *chinmo-lacZ, elav-Gal4>UAS-abd-A*. ABLKs coexpress *chinmo-lacZ* in both genotypes. See white arrows in B,E. Only a few stacks are shown in E in order to simplify the image. (**C,F**) Expression of Lk (red) and Br-C (blue) in *wild-type* and *elav-Gal4>UAS-abd-A*. ABLKs do not coexpress Br-C in any of the genotypes. White arrows point to a single ABLK (C) or a cluster of them (F). All the ventral nerve cords of A-F figures correspond to 18h AEL embryos.

As mentioned before, we did not detect any morphological difference among the ectopic ABLKs found in the *abd-A* misexpression experiment. However, we wondered if there was any molecular marker that was differentially expressed between the eABLKs, and what we assume were, the pABLKs. We stained for expression of Chinmo and Broad- Complex (Br-C), which are pan-lineage markers for early and late neuronal identity, respectively (Maurange et al., 2008). Chinmo is expressed in neurons generated during embryonic to mid-larval stages, whereas Broad is present in neurons produced from mid-larval stages onwards. We found that, in *wild-type* and in ganglia misexpressing *abd-A,* both bona fide and ectopic ABLKs were Chinmo positive and lacked expression of Broad- Complex (Fig. R.29B, C, E and F). In addition, all ABLKs found in adults, embryonic and postembryonic ABLKs, behaved in the same way (data not shown).

6.11. Abd-B represses the expression of Leucokinin.

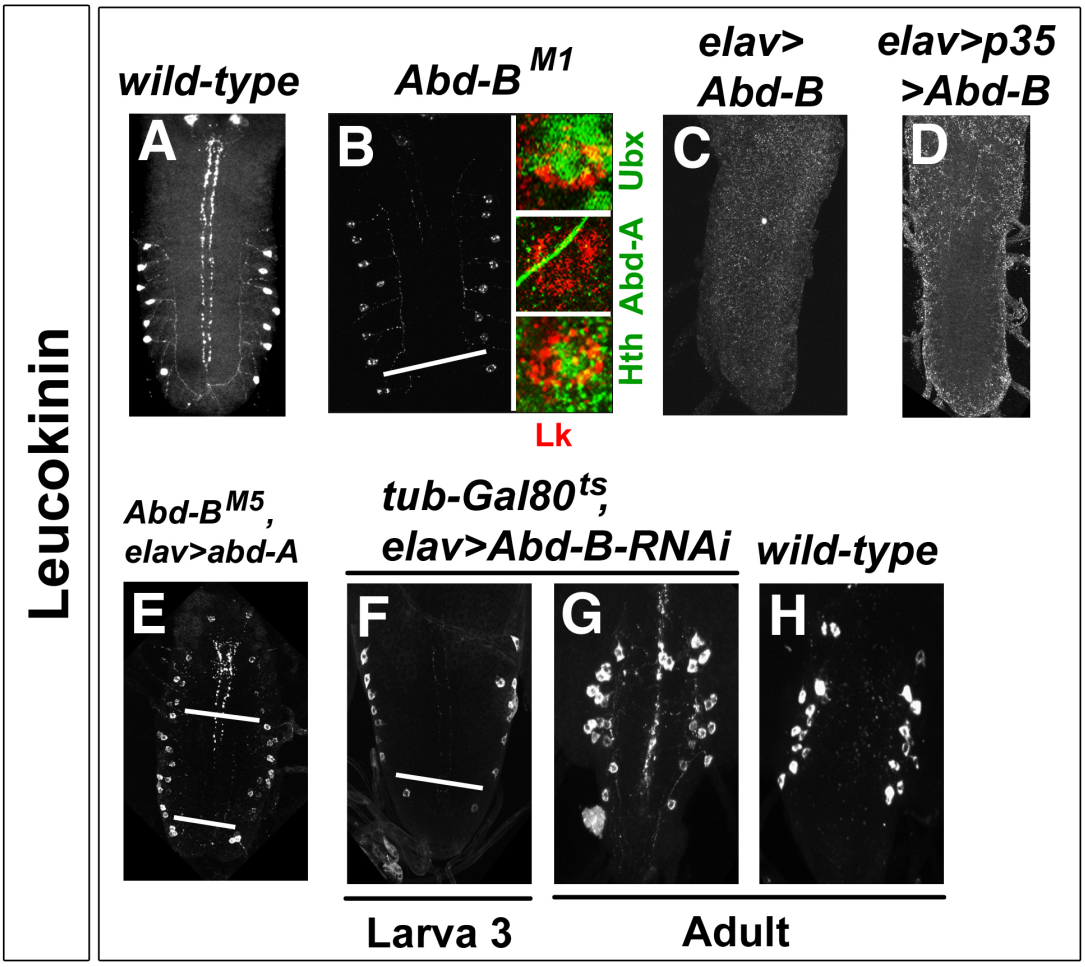
We next assessed the role of Abd-B in patterning the most posterior abdominal segments. In *abd-A* misexpression, the extra ABLKs were mostly restricted to the most anterior abdominal segments (A1-4). This suggested a role of Abd-B in repressing the ABLK fate in the most posterior region, since Abd-B is expressed segments A4-9 (Celniker et al., 1990; Sanchez-Herrero, 1991) (Fig. R.24A).

Indeed, as mentioned before, we observed an extra ABLK of the corresponding A8 hemisegment in individuals' mutant for *Abd-B* (*Abd-B*^{M1} χ =7.98 ±0.15*, Fig. R.30B, I and J). Under these conditions, we checked for the expression of Ubx and Abd-A and found that only Ubx was expressed in this new ABLK (Fig. R.30B). It has been shown that, in the nervous system of *Drosophila*, the microRNA *iab8* works together with Abd-B to repress *abd-A*;

therefore the absence of Abd-A in segment A8 means that the microRNA *iab8* is not altered in this genotype and it is still able to repress *abd-A*. On the other hand, under loss of Abd-B Ubx becomes derepressed (Gummalla et al., 2012).

Conversely, all ABLKs disappeared when *Abd-B* was misexpressed (*elav-Gal>UAS-Abd-B* χ =0.02 ±0.13*, Fig. 30C, I and J). To check if this phenotype was due to the already reported proapoptotic role of *Abd-B* (Miguel-Aliaga and Thor, 2004), we performed the same experiment but inhibiting apoptosis (*elavG4>UAS-p35 UAS-AbdB*), and obtained the same result (χ =0.08 ±0.28*, Fig. 30D, I and J). This indicated that Abd-B does not promote programed cell death of ABLKs, but it either represses the ABLK fate or the expression of Lk.

To further corroborate this conclusion, we overexpressed *Abd-B-RNAi* from first instar larva (*tub-Gal80^{ts}*, *elavG4>UAS-Abd-B-RNAi*, see experiment 6 in Materials and Methods) and observed the correspondent ABLK of A8 in third instar larva individuals (χ =7.56 ±0.51*, Fig. 30F, I and J). This suggests that Abd-B represses the expression of Lk but not the fate of the ABLK, thus, as soon as it was removed from the neuron, Lk was de-repressed. We also checked for the expression of Lk in adult stages and found that the number of ABLKs rose up to 15 per hemiganglion, which probably corresponds to two ABLKs, the embryonic and postembryonic ones, from segments A1-8 (χ =12.69 ±1.32*, Fig. R.30H, I and J). This set of results strongly suggest that Abd-B is repressing the expression of LK in the ABLK of segment A8 during the embryonic neurogenesis, whereas it does so in the ABLKs from A5-8 during the larval neurogenic period. To further test this hypothesis, we overexpressed *abd-A* in *Abd-B* mutant (*Abd-B^{M1}, elav-Gal4>UAS-abd-A);* we reasoned that if pABLKs appear in the most anterior abdominal segments in first instar larva upon *abd-A* misexpression, loss of Abd-B repression should generate extra pABLKs in all segments, and this was indeed what we found (χ =13.29 ±3.79*, Fig. R.30E, I and J), there were clusters of 2-3 ABLKs in segments T1-A8. We assume that these are the embryonic and postembryonic ABLKs.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Lk
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E
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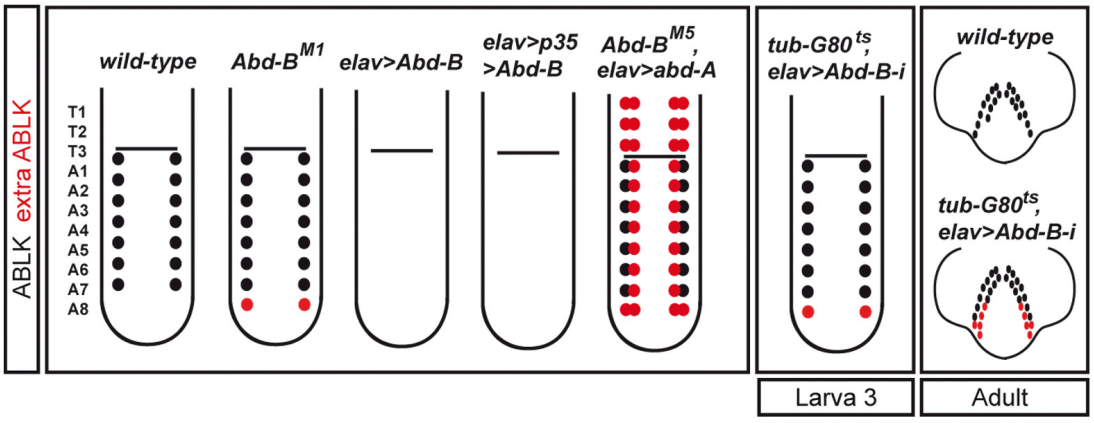
Larva 3
Adult

I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wild-type
tub-G80 ts, elav>Abd-B-i
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A8
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Adult

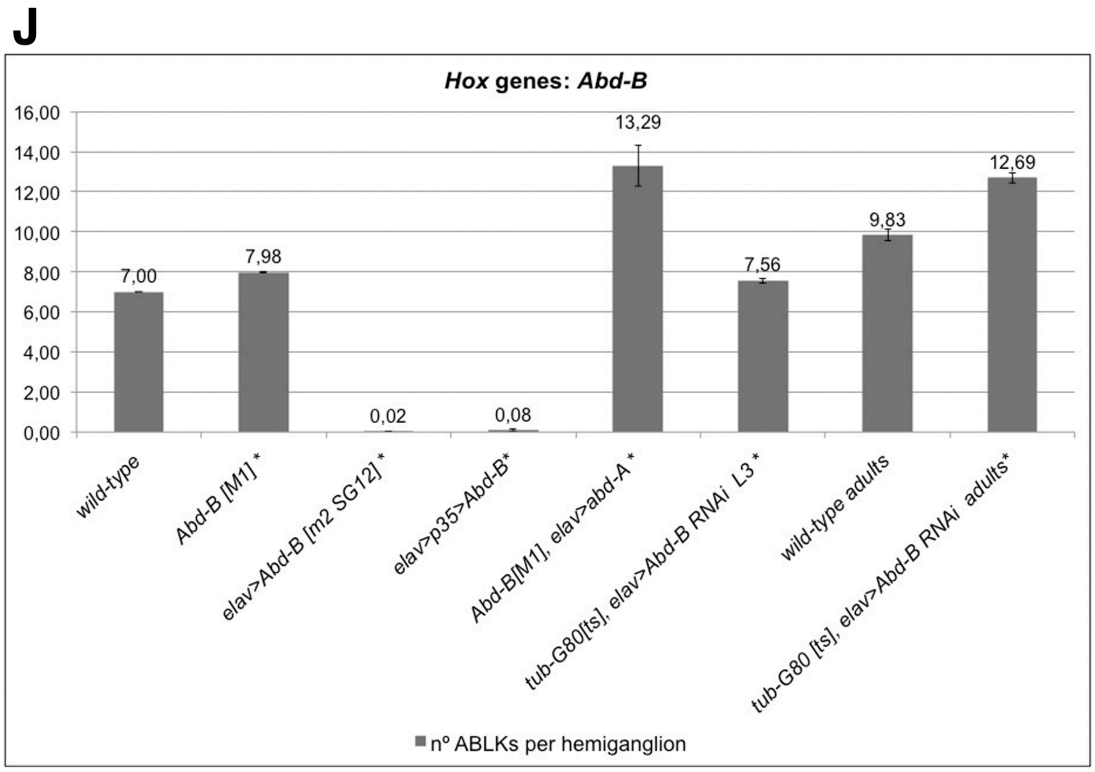


Fig. R.30. *Abd-B* represses the expression of Lk. (**A-E**) Expression of Lk in *wild-type* (A), *Abd-B^{M1}* (B), *elav-Gal4>UAS-Abd-B* (C), *elav-Gal>UAS-p35 UAS-Abd-B* (D) and *Abd-B^{M5}, elav-Gal4>UAS-abd-A* (E) VNCs of 18h AEL embryos. (B) The correspondant ABLK of A8 shows up when *Abd-B* is lost. White bar separates the segments A7/A8. Expression of Lk (red) and Ubx, Abd-A and Hth (green) in the ABLK of A8 is shown in lateral insets. This ABLK expresses Ubx and Hth, but not Abd-A. (C) ABLKs are lost upon misexpression of *Abd-B*. (D) The role of *Abd-B* in the specification of ABLKs is not to promote apoptosis; ABLKs are not rescued when both *Abd-B* and the caspase inhibitor *p35* are misexpressed. (E) Clusters of ectopic ABLKs are found in segments T1-A8 when *abd-A* is misexpressed in an *Abd-B* mutant background. White bars separate the segments T3/A1 and A7/A8. This suggest that *Abd-B* represses the ABLK fate in segment A8 in embryonic neurogenesis and in segments A5-8 during larval neurogenesis. (**F,G**) Expression of Lk in *tub-Gal80^{ts}, elav-Gal4>UAS-Abd-B-RNAi* in VNCs of third instar larvae (F) and adults (G). *Abd-B* was knocked down from first instar larvae onwards. (F) An ABLK appears in A8. White separate the segments A7/A8. (G) The number of ABLKs rose to 15 per hemiganglion, which probably corresponds to two ABLKs per hemisegment in A1-8. (**H**) Expression of LK in a VNC of adult stage. (**I**) Schematic summarizing the phenotypes observed. (**J**) Histogram depicting the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type.*

6.12. *homothorax/Meis1* is not required for ABLK specification.

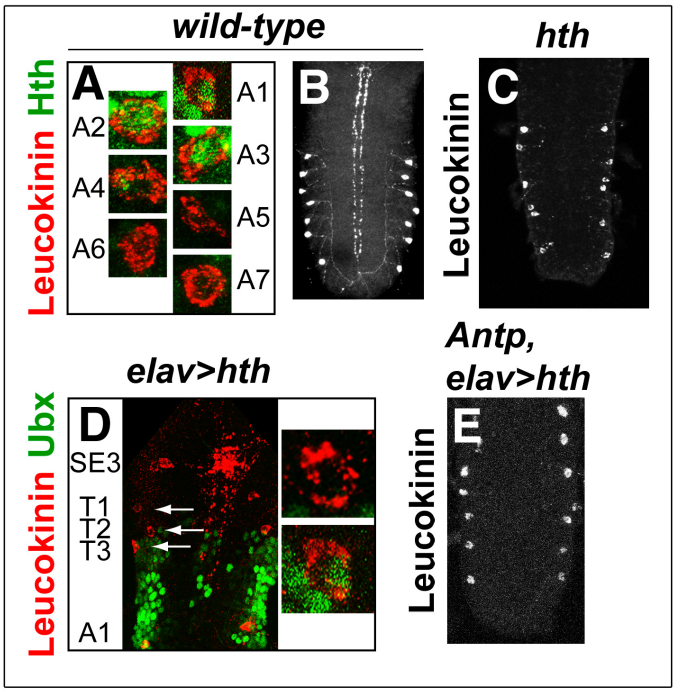
Hox proteins achieve target-binding specificity through the action of cofactors. These proteins bind DNA cooperatively with Hox proteins and thereby help them with DNA-binding site selection. In *Drosophila*, two Hox cofactors have been described: Extradenticle (Exd)/Pbx (Pbx) and Homothorax (Hth)/Meis1 (Mann and Affolter, 1998).

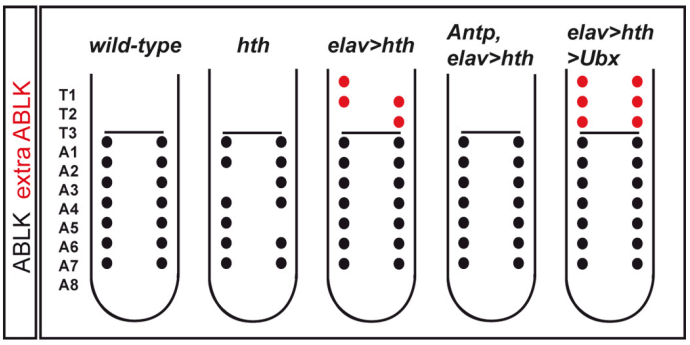
hth/Meis1 gene encodes a homeodomain protein that binds to Exd and facilitates its entrance to the nucleus (Abu-Shaar et al., 1999; Azpiazu and Morata, 1998; Kurant et al., 1998; Ryoo et al., 1999). Recently, it has been described that among the six different isoforms of Hth, not all of them translocate Exd into the nucleus (Corsetti and Azpiazu, 2013). Mutations in either *hth* or *exd* strongly affect Hox gene function. It has been suggested that Hth binds to DNA together with Hox/Exd heterodimers to form Hth/Hox/Exd trimer complexes (Ryoo et al., 1999). Although *exd* is expressed both maternally and zygotically, *hth* does not appear to be maternally inherited so that its loss of function resembles the complete absence of *exd* (Peifer and Wieschaus, 1990; Rauskolb et al., 1993; Rieckhof et al., 1997). Since we found that Ubx, Abd-A and Abd-B participated in the specification of ABLKs, we analysed the role of Hth in this process.

First, we saw that Hth was expressed in ABLKs of segments A1-3 and was absent in segments A5-7. ABLK of segment A4 exhibited an intermediate situtation since co-expression was only found in some ganglia (60% out of a total of 20 hemisegments A4 scored, Fig. R.31A).

Interestingly, the expression pattern of Hth did not change in *Abd-B* mutants (*Abd-B^{M1}*), but the extra ABLK observed in segment A8 expressed Hth. (Fig. R.30B). It is known that Abd-A downregulates *hth* expression (Kurant et al., 1998); thus, this result is not surprising as the ABLK in segment A8 expresses Ubx but not Abd-A. (Fig. R.30B).

To test for a requirement of Hth in ABLK specification, we looked at the expression of LK in *hth* mutants *(hth^{5E04}/hth^{6158})* and detected a slight decrease in the number of ABLKs (χ =5.44 ±1.20*, Fig. R.31C, F and G). Likewise, the expression of other neuropeptides such as FMRFa, Nplp1 and CCAP was lost (Karlsson et al., 2010) (M. Moris.-Sanz., unpublished results).





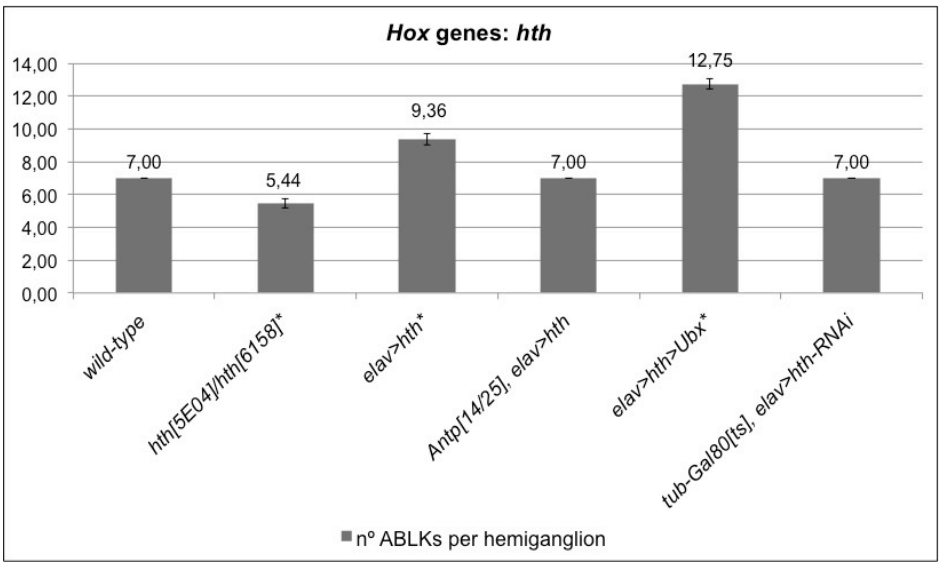


Fig. R.31. *hth* is not required for ABLK specification. (**A**) Expression of Lk (red) and Hth

(green) in *wild-type*. Individual ABLKs of segments A1-A7 are shown in the insets. (**B,C**) Expression of Lk in *wild-type* (B) and *hth*$^{5E04/6158}$ (C); there is a slight decrease in the number of ABLKs. (**D**) Expression of Lk (red) and Ubx (green) in *elav-Gal>UAS-hth.* The area shown corresponds to the third subesophagic segment (SE3), the three thoracic segments (T1,T2 and T3) and first abdominal segment (A1). White arrows indicate the new ABLKs found in the thoracic segments. Higher magnification views of ABLKs from thoracic segments T2 and T3 are shown to the right of the figure. (**E**) Expression of LK in *Antp, elav-Gal4>UAS-hth*; there is no alteration in the pattern of ABLKs. All VNCs of figures A-E corresponds to 18h AEL embryos. (**F**) Schematic summarizing the phenotypes observed. (**G**) Histogram depicting the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type.*

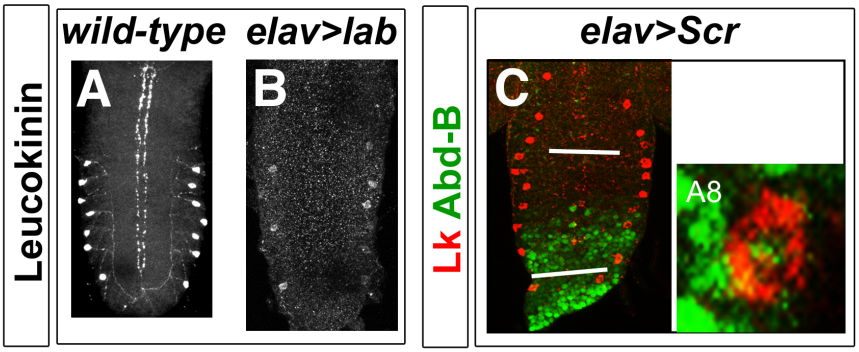
Strikingly, misexpression of *hth* (*elav-Gal4>UAS-hth*) produced extra ABLKs in all thoracic segments (χ =9.36 ±1.56*, Fig. R.31D, F and G), but not in the more anterior segments of the CNS, i.e. in the subesophagic segments. We found that Ubx was expressed in these ectopic ABLKs of segment T3, but was absent in the corrresponding ABLKs of segments T1-2 (Fig.R.31D). In these later segments, Hth would be acting either alone or in combination with the Hox protein present there, Antp (#374: Hirth et al 1998). To distinguish between these two possibilities we misexpressed *hth* in an *Antp* mutant background (*Antp*^{25}*, elav-Gal4>UAS-hth*) and observed that under these conditions there were no extra ABLKs (χ =7.00 ±0.00, Fig. R.31E, F and G).

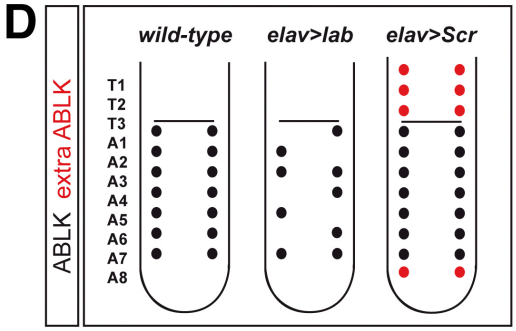
We next wondered whether the combination of Hth and other Hox protein expressed in the trunk, i.e. Ubx (*elav-Gal4>UAS-hth UAS-Ubx*), would be enough to trigger the formation of ABLKs in the more anterior or posterior segments. The phenotype we found was similar to misexpressing *Ubx* or *hth* alone, although the penetrance was higher and ocasionally some ABLKs appeared duplicated (χ =12.75 ± 2.22*, Fig. R.31F and G).

Finally, we tested if Hth was also required for the maintenance of Lk, as it does Ubx and Abd-A. We knocked-down *hth* in first instar larvae and looked for the expression of Lk in third instar larvae (*tub-Gal80*^{ts}, *elav-Gal4>UAS-hth-RNAi* (long-form), experiment 7 in Materials and Methods). We did not detect changes in the ABLKs expression pattern (χ =7.00 ± 0.00, Fig. R.31G), although we were able to detect some anti-Hth staining, maybe this could be the reason of the lack of phenotype.

6.13. Scr can activate Leucokinin expression.

Although gene members of the Antp-complex are expressed in the subesophagic and thoracic segments, where ABLKs are not present, we wanted to test their ability to promote ABLK specification. To do that, we studied the effects of their misexpression. We found that the number of ABLKs was reduced in *elav-Gal4>UAS-lab* (χ =4.44 ± 1.78*, Fig. R.32B, D and E) and increased in *elav-Gal4>UAS-Scr* (χ =8.58 ± 1.61*, Fig.R.32C, D and E). The rest of the misexpression experiments did not produce any significant changes in the pattern of ABLKs (Fig. R.32E). The increase in the number of ABLKs in the *Scr* misexpression experiment consisted of the appearance of ABLKs in segments T1-3 and A8, although the penetrance of this phenotype was not complete. This result suggests that first, Scr would be able to activate ABLK fate in thoracic segments, either acting with Hth/Antp or activating LK fate by itself. On the other way, the fact that the ABLK from A8 only appears when *Abd-B* was inactivated, suggests that *Scr* misexpression is able to counteract the role of Abd-B within this neuron.





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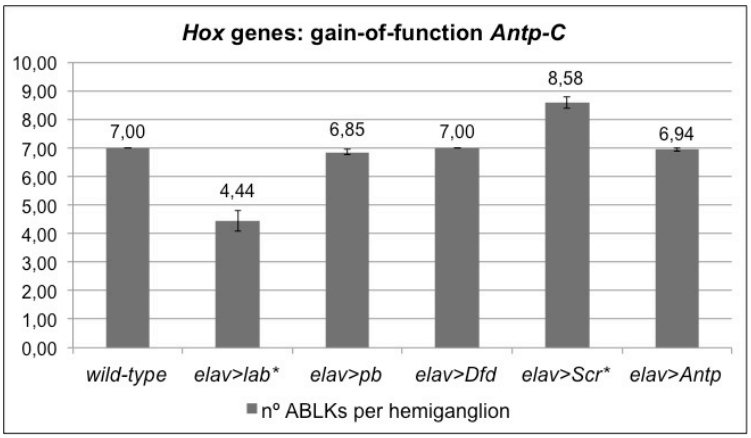

Fig. R.32. Scr can activate Lk expression. (**A,B**) Expression of Lk in *wild-type* (A) and *elav-Gal4>UAS-lab* (B). The number of ABLKs significantly decreases upon misexpression of *lab*. (**C**) Expression of Lk (red) Abd-B (green) in *elav-Gal4>UAS-Scr*. ABLKs appear from T1 to A8 segments. A higher magnification view of one ABLK in the A8 segment is shown on the right. Bars separate the T3/A1 and A7/A8 segments. All VNCs from figures A-C are of 18h AEL embryos. (**D**) Schematic summarizing the phenotypes observed. (**E**) Histogram depicting the number of ABLKs per hemiganglion found in the different genotypes. Means are shown at the top of each bar. An asterisk next to the name of the genotype indicates that there were significant differences compared to *wild-type.*

7. Discussion

7.1.Identification of embryonic lineages in *Drosophila.*

Although the type and number of cells generated by each embryonic NB lineage has been extensively studied (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997), the assignment of a particular neuron with a specific terminal identity to its appropriate lineage and progenitor NB has been addressed in very few cases. For that, to specifically label a particular neuron, it was used either molecular markers expressed in postmitotic neurons such as Even-skipped (Eve), Cut, Zn finger homeodomain 1 (Zfh1), Islet, HB9, En, Zn finger homeodomain 2 (Zfh2); or the expression of neuropeptides such as FMRFa, or Neuropeptide-like precursor 1 (Nplp1). This also has been possible due to the availability of lineage markers such as *eagle* for NBs 2-4, 3-3, 6-4 and 7-3 and *lbe(K)* for NB5-6. The cases in which specific neuron types have been linked to their lineages are shown in the following table (Table D.1).

Table D.1: List of specific cell types identified in the embryonic *Drosophila* CNS.

Name of NB	Progeny generated	References
NB1-1A	-aCC (anterior Corner Cell). -pCC (posterior Corner Cell). -A-, B-SPG and LV-SPG (A, B and subperineurial glia).	(Beckervordersandforth et al., 2008; Ito, 1995; Klambt and Goodman, 1991).
NB1-3	- M-ISNG (Medial- intersegmental nerve root glia). - MD-SPG (Medial dorsal subperineurial glia).	(Beckervordersandforth et al., 2008; Ito, 1995).
NB3-1	- RP ("raw prawns") motoneurons: RP1,3,4 and 5.	(Bossing et al., 1996; Landgraf et al., 1997; Tran and Doe, 2008).
NB3-3T	- 6 EL (Eve-lateral) cluster of interneurons.	(Schmid et al., 1999; Schmidt et al., 1997; Tsuji et al., 2008).
NB3-3A	- 11 EL (Eve-lateral) cluster of interneurons.	(Schmid et al., 1999; Schmidt et al., 1997; Tsuji et al., 2008).
NB4-2	- RP2 motoneuron.	(Yang et al., 1997; Yang et al., 1993; Yeo et al., 1995).

NB5-3	- Capa expressing neurons.	(Gabilondo et al., 2011).
NB5-5A	-Abdominal leucokinergic neurons (ABLKs).	This thesis (Benito-Sipos et al., 2010).
NB5-6T	- Ap cluster that comprises Ap1/Nplp1, Ap2, Ap3 and Ap4/FMRFa. - DL-SPG (Dorsal lateral), MV-SPG (Medial ventral) and VL-SPG (Ventral lateral).	(Baumgardt et al., 2009; Beckervordersandforth et al., 2008; Ito, 1995; Karlsson et al., 2010).
NB6-4T	-MM-CBG (Medial most-cell body glia).	(Beckervordersandforth et al., 2008; Ito, 1995).
NB6-4A	- Medial-CBG and MM-CBG.	(Beckervordersandforth et al., 2008; Ito, 1995).
NB7-1	- U1 to U5 motoneurons.	(Pearson and Doe, 2003).
NB7-3	-GW motoneuron. - EW1,2 and 3 interneurons.	(Karcavich and Doe, 2005; Lundell and Hirsh, 1998).
NB7-4	-D-CG (Dorsal channel-glia), Ventral-CG. - Lateral-CBG. -Medial lateral-SPG. - Lateral-ISNG.	(Beckervordersandforth et al., 2008; Ito, 1995).

So, only part of the progeny of 12 out of 30 NB lineages is known. The study of these lineages allows using them as model systems to investigate several processes. For instance, the mentioned lineages have been widely employed to shed light on temporal gene transitions and specification, NB competence, modes of NB division, entry into quiescence, the role of Notch signalling pathway and of Hox proteins in the CNS.

Moreover, from the above-mentioned lineages, only three have been characterized completely in terms of the number of neurons generated for the case of NB7-3 (Karcavich and Doe, 2005), but not in terms of all their neural identities. These latter are the thoracic and abdominal counterparts of NB3-3 (Tsuji et al., 2008) and the ones of NB5-6 (Baumgardt et al., 2009; Karlsson et al., 2010). As mentioned before, this have been possible due to the discovery of specific lineage markers for each of them, *eg* and *lbe (K),* respectively. Thus, it is likely that the further characterization of *Drosophila* embryonic lineages will provide new tools for the study of fundamental questions in developmental neurobiology.

Here we have assigned the abdominal leucokinergic neurons (ABLKs) to the lineage of NB5-5A. However, the lack of molecular markers avoided us to further identify other members of this lineage, and define the specific birth-date of ABLKs within the lineage. It has been described that NB5-5A generates 6-9 neurons in *wild-type* embryos, whereas it produces 12-15 neurons in *Df(3L)H99* individuals (Rogulja-Ortmann et al., 2007). This data indicate that would be at least 6 rounds of divisions, considering that NB5-5A divides canonically producing a GMC that generates two daughter cells.

7.2. Temporal transitions in embryonic NBs in *Drosophila*.

Not all NBs undergo the full series of transcription factors. It has been shown that some NBs delaminate already expressing Cas, such as NB2-1, 3-4, 5-1 and 6-1 (Doe, 1992). NB3-3 starts the temporal series with Kr (Tsuji et al., 2008), whereas other NBs delaminate already expressing Svp, which downregulates *hb* activity, these are: NB 4-1, 4-3, 5-1, 5-2 and 7-2 (Doe, 1992).

About the temporal sequence of NB5-5A, we detected Pdm expression by its time of delamination (late stage 11:7h of embryonic development). Individual Pdm windows usually persist for just one NB division (NB3-1: (Tran and Doe, 2008) and NB4-2: (Yang et al., 1997)), although coexpression with Cas during the next NB division has also been reported (NB3-3: (Tsuji et al., 2008), NB5-6: (Baumgardt et al., 2009) and NB7-1: (Pearson and Doe, 2003)). We assume that during this first division the NB only expresses Pdm. Cas is detected at stage 12 (7-9h of embryonic development), since embryonic NB divisions normally take around 1h, two Cas-expressing NBs might be generated during this time. Grh expression starts at stage 13 (9-10h of embryonic development), when it is coexpressed with Cas. According to the loss- and gain-of-function and colocalization experiments, we propose that ABLKs are generated within this Cas/Grh temporal window. Since embryonic NB5-5A is one of the three abdominal embryonic NBs that will continue proliferating in larval stages, we assume that NB5-5A will maintain this Cas/Grh temporal profile till it stops dividing and enter quiescence by the end of embryonic development.

7.3. Role of temporal genes in neural specification.

Temporal genes are called so because they are expressed at different times during development and specify the identity of a particular set of progeny within a given lineage. In our case, we showed that ABLKs expressed Cas and *grh-lacZ* and were lost in these mutants. Consequently, we found ectopic ABLKs when misexpressing *cas,* and *grh* together with *dimm*. These findings indicate that ABLKs are generated by NB5-5A when expressing Cas/Grh. Apart from acting as a temporal gene; Cas has also been proposed to be a switching temporal factor needed to close the third temporal identity window of NB3-1 (Tran and Doe, 2008). However, in NB5-5A our results indicate that Cas acts as a temporal factor needed to specify the ABLKs, considering the results of the loss- and gain-of-function experiments, as well as its colocalization in the ABLKs. We also obtained a slight decease in ABLKs pattern in *Kr* mutants; we do not have an explanation for this result. It is possible that lack of *Kr* could affect the activation of *cas*, however, it has been shown that only the neurons generated from a *Kr*-expressing NB are lost when this factor is mutated (Isshiki et al., 2001; Novotny et al., 2002).

In principle, temporal factors cross-regulate each other, however, such cross-regulations do not appear to be decisive because loss of Hb, Kr or Pdm only leads to one temporal identity being skipped, rather than a blockade of the subsequent temporal factors. Thus, it might exist a mechanism, so far unknown that will act as timer defining the different temporal transitions (Jacob et al., 2008). It has also been proposed that this timely expression of temporal factors is the result of default transcription of *pdm* and *cas* in NBs (Maurange, 2012). However, none of these hypotheses have been proven to date. For that, it will be determinant to characterize further NB lineages in which to address these questions.

On the other hand, we also detected expression of *svp-lacZ* in ABLKs. Our results suggest that this corresponds to the second pulse of Svp detected in some NBs at late embryonic stages (Benito-Sipos et al., 2011; Kohwi et al., 2011; Tsuji et al., 2008). In the lineages in which broad Cas windows have been studied, NB3-3 and NB5-6T, Cas expression takes place in two bursts separated by a non-Cas NB. Moreover, the subsequent second pulse of Svp accompanies this second burst of Cas. Thus, it is tempting to speculate that such a mechanism also occurs in NB5-5A; but since the absence of Cas in the NB lasts so little in time, just for one division (1h approximately), we were not able to detect it.

7.4. Role of Notch signalling pathway in neural specification.

In both vertebrates and invertebrates, Notch signalling pathway is widely employed during nervous system development, particularly, when cells choose between two alternative fates, a process called binary fate decision. During early stages (neural versus epidermal fate), Notch signalling leads to inhibition towards neural fate. However, it then acts as a binary switch in cell fate decisions between different subtypes of neural cells, being instructive for one fate and inhibitory for the other. (Reviewed in (Cau and Blader, 2009; Udolph, 2012)).

Here, we have shown that the ABLK and its sibling are equivalent cells committed to die, and activation of the Notch pathway prevents its death. A similar situation has been described for specification of the aCC/pCC neurons in the NB1-1 lineage in grasshopper, in which the sibling cells start as equivalent neurons and interaction between them lead to different fates (Kuwada and Goodman, 1985). By contrast, in NB7-3, lack of N signalling is required for the differentiation of the EW2 and EW3 interneruons, whereas activation of N in their sibling cells leads to their apoptosis (Karcavich and Doe, 2005; Lundell et al., 2003). In our work, activation of the N pathway, or inhibition of PCD is enough to generate two ABLKs per hemisegment. NB5-5A gives rise around 6-9 neurons, whereas it produces 12-15 neurons in *Df(3L)H99* individuals (Rogulja-Ortmann et al., 2007), which fits with our findings that most GMCs of the lineage give rise to a neuron and a sibling cell that dies by apoptosis. We conclude that in this lineage, N signalling does not play an instructive role but allows the commitment to a specific fate decision by regulating the competence to activate PCD.

7.5. A model for the specification of ABLKs.

We have identified a set of genes that are required for the specification of ABLKs. These are *nab, sqz, klu, cas, grh, jumu, kni* and *tsh*.

The *jumu* phenotype was expected given that it has been shown that Jumu is required in the NB4-2 lineage for appropriate segregation of Numb in asymmetric cell divisions (Cheah et al., 2000). In *wild-type* conditions, GMC-2 of NB4-2 lineage generates the motoneuron RP2, which is Numb positive and therefore Notch off, whereas its sibling cell does not inherit Numb

and activates N signalling. In *jumu* mutants they found that both daughter cells adopted the RP2 sibling cell fate, both inherited Numb. Likewise, in *jumu*, both daughter cells of NB5-5A lineage adopt the fate of the ABLK sibling cell, which possesses Numb, and consequently, we detect a decrease in the number of ABLKs.

About the specific roles of *kni* and *tsh* in ABLK specification, we cannot provide anymore data than what has already been mentioned. Neither of these genes have been addressed previously in the CNS of *Drosophila*, therefore, further experiments concerning these genes are likely to contribute valuable knowledge. *kni* is a gap gene, same as *hb* and *Kr*, which function as temporal genes in embryonic development of the CNS. About *tsh*, as we indicated in the results, it has been described that it controls the expression of the Lk receptor (Lkr), however, this would not have to necessarily affect ABLK specification. Yet, since the receptor of Lk has been found in ABLKs themselves, it has been suggested that they regulate their own activity through autocrine signalling (Okusawa et al., 2014). To check this possibility, it will be needed to know if disruption of Lkr is able to alter the pattern of expression of ABLKs.

The genes with which we have worked more have been *cas, grh, nab, sqz* and *klu*. It has been shown that in *cas* mutants, it disappears the expression of *nab* mRNA (Clements et al., 2003), *sqz* mRNA (Baumgardt et al., 2009) and Klu (this thesis). However, Tsuji et al. (Tsuji et al., 2008) showed that Cas did not directly activate *nab* and *sqz*, but it did it through repression of Pdm. Taken together our results, we propose a model in which *cas* will be upstream of the ABLK genetic code specification activating *sqz, nab, grh* and *klu*. In such a model, Sqz would act as a repressor, either direct or indirect, upon ABLK specification. Nab would bind Sqz and block its repressive activity (Fig. D.1).

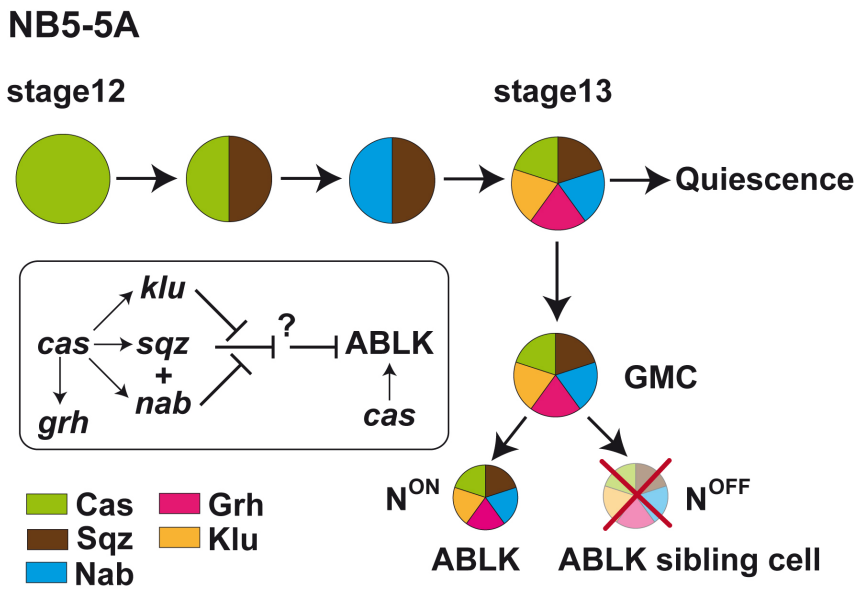

Fig. D.1. Schematic showing the proposed model for ABLK specification. NB5-5A starts expressing Cas at stage 12 and would pass through a Cas, Cas/Sqz, Sqz/Nab, Cas/Sqz/Nab/Klu/Grh rounds of divisions. Our data indicate that ABLKs arise from this latter round of division; both the ABLK and its sibling cell are equally competent to differentiate into Lk fate but Notch signalling needs to be activated to repress programmed cell death. After generating the embryonic ABLKs, NB5-5A most likely enters quiescence and will produce the postembryonic ABLKs during the larval neurogenesis. It is also shown a model of the relationships between the genes involved in this process. Cas activates *sqz, nab, grh* and *klu*. Sqz represses either directly or indirectly the ABLK fate (represented by a question mark); Nab and Klu block this inhibition. Moreover, Cas would also be downstream of these Klu and Nab actions. Abbreviations: GMC, ganglion mother cell.

There are other 2 lineages (NB3-3 and NB5-6) in which the activities of Cas, Nab and Sqz have been studied (Baumgardt et al., 2009; Tsuji et al., 2008). There, they described an initial Cas expression window in the NB that activated both *sqz* and *nab*. However, these two activations were slightly separated in time. Sqz expression always preceded the one of Nab, resulting in a narrow window of time in which the NB only expresses only Cas/Sqz but not Nab. With this in mind, and according to our results, we propose that Sqz would be repressing Cas. Later on, Nab would be active and form a complex with Sqz that will block its repressive action on Cas. Thus, the sequence in the successive rounds of divisions of NBs would be as follows: Cas, Cas/Sqz, Nab/Sqz, Cas/Nab/Sqz. We propose that ABLKs would come from the Cas/Nab/Sqz NB. This model fits with most of the results obtained. Moreover, we add Klu, which was not studied before, but our data indicate that Cas activates its expression and it is required for ABLKs specification. Additionally, our data suggest that represses Sqz, although

we do not know at what level. In addition, the fact that we did not find rescue when misexpressing *nab* in *cas* mutants, indicates that Cas plays additional roles in ABLK specification, than just activating *nab*. Also, misexpression of *cas* was able to rescue *nab* and *klu* phenotypes, which places it downstream these genes, more likely in the post-mitotic neuron. It has already been suggested that temporal and subtemporal genes may act at differently in the NB and progeny (Jacob et al., 2008).

7.6 The onset of leucokinin expression takes place at two different stages in development.

Here we have shown that ABLKs are generated during the two neurogenesis of the fly. Embryonic ABLKs are produced in the embryonic neurogenesis and postembryonic ABLKs arise in the larval one. However, their terminal differentiation, that is, expression of the neuropeptide Lk is delayed with respect to their birth time. In our case, eABLKs are born around embryonic stages 13-15 and Lk is first detected at 18h AEL. Likewise, pABLKs are produced from second instar larvae and Lk starts to be expressed from pupal stages onwards.

There are other examples in which the expression of the neuropeptide is delayed; in FMRFa and CCAP neurons (Schneider et al., 1993; Veverytsa and Allan, 2012). It is not known the functional relevance of this temporally tuned neuronal differentiation, but what our results suggests is that, independently of the factors required for the onset of Lk expression, these regulators have two bursts of expression, in the late embryo and late pupa. In this way, when pABLKs are mistemporally generated in the embryo upon *abd-A* misexpression experiment, all express Lk at the same time. Ecdysteroid hormone titers show two peaks, in late embryo and pupa (Richards, 1981), and it has been shown that ecdysone signal induces terminal differentiation of some of the CCAP-expressing neurons in pupa (Veverytsa and Allan, 2012). In this case, the authors demonstrated that although all CCAP neurons had an embryonic origin, a subset of them did not start their differentiation programme, i.e. expression of peptides and formation of axonal projections, till pupariation. In our case, we have not succeeded, so far, in the identification of the factors involved in the terminal differentiation of ABLK neurons.

7.7. Embryonic and postembryonic ABLKs are generated by the same neuroblast.

We have shown that NB5-5A is the progenitor neuroblast of eABLKs. Our findings indicate that this embryonic NB enters quiescence at the end of embryogenesis and resume proliferation as the postembryonic vl NB that generates the pABLKs. A previous study showed that pNBs are not an independent subset of the NBs generated in the embryo that remain dormant during embryonic neurogenesis and only proliferate in larval stages (Prokop and Technau, 1991). To our knowledge, this is the first reported case in which the same NB specifies a cell fate at different developmental stages.

After embryonic neurogenesis, there is a major segment-specific elimination of NBs in the CNS of *Drosophila*. Most of the NBs from thoracic segments (>20/30 NBs per hemisegment) survive and enter quiescence, whereas only 3 NBs do so in the central abdomen segments (A3-7). The identities of the subsequent pNBs have not been addressed completely. In thoracic segments, only 10 out of the 24 of the adult-specific lineages have been associated to their embryonic NB (Truman et al., 2004). More recently, in order to unambiguously identify these pNBs and their progeny, a gene expression map has been established according to the expression of 15 transcription factors (Lacin et al., 2014). In subesophagic segments, only 13 paired lineages and one unpaired have been found (Kuert et al., 2014); yet, the embryonic identities of these NBs were not addressed. In central abdominal segments, the three pNBs are identified and named according to the position they occupy in the VNC; vm, vl and dl. Their identities respect to their embryonic equivalents had not been pinpointed so far, but had the estimated progeny generated by each of them. Both vm and vl pNBs give rise to an average number of four neurons (Truman and Bate, 1988), while dl pNB produces around 9 neurons (Bello et al., 2003; Truman and Bate, 1988). Thus, the identification of these larval lineages and their embryonic counterparts will provide new tools in which to study how eNBs stops dividing at the end of the embryonic period and how quiescence is regulated both temporally and spatially.

Temporal factors define the competence to activate specific cell fates during development (Maurange, 2012), and in essence, we show that when eNB5-5A resumes proliferation after the quiescent period, it maintains the same competence window. Unfortunately, lineage markers that would allow us to follow the complete lineage of this NB

throughout development are not available to date, but we assume that the eABLK and the pABLK are, respectively, the last and the first neurons in this lineage generated before and after quiescence.

7.8. Ubx, abd-A and Abd-B sculpt the pattern of ABLKs in embryonic neurogenesis.

The segment-specific appearance of ABLKs made us wonder about the roles of the *Hox* genes in this outcome. Our findings point towards a complex interplay between the members of the Bx-C to regulate the appropriate pattern of expression of ABLKs. (Fig. D.2)

We found that Ubx was expressed in ABLKs from segments A1-7 and Abd-A in A2-7 ABLKs. All ABLKs were lost when these genes were removed, and we found ectopic ABLKs in thoracic segments upon individual misexpression of these genes. Also, in Abd-B mutants we observed an extra ABLK in A8, while its ectopic expression eliminated them all. Together, these findings indicate that Ubx and Abd-A are redundantly required to specify the ABLKs in A2-7 segments, whereas Ubx is needed to do so in the first abdominal segment. (Fig. D.2).

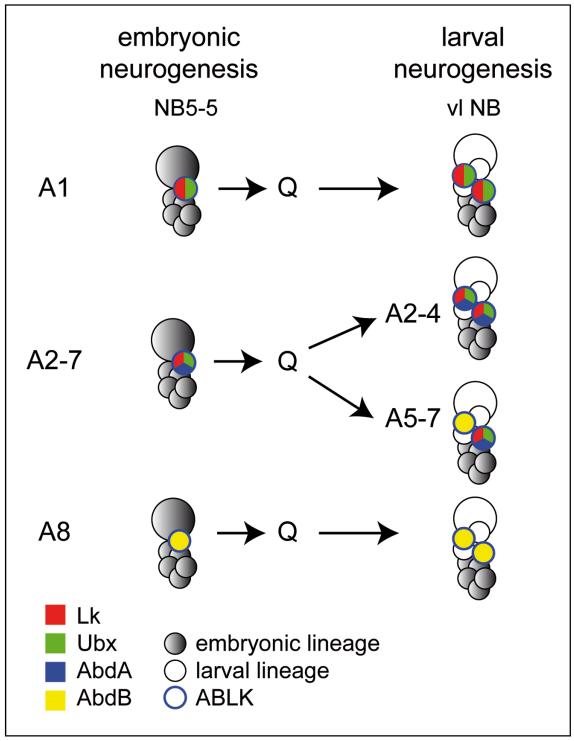

Fig. D.2. Model of ABLK specification by *Hox* genes. Schematic of the mechanism employed by *Hox* genes to regulate the segment-specific appearance of ABLKs. NB5-5A generates the embryonic ABLKs (eABLKs), enters quiescence and then transforms into the ventrolateral postembryonic NB (vl pNB) to give rise to the postembryonic ABLKs (pABLKs). Ubx and Abd-A are expressed and redundantly required to specify the eABLKs of A1-7 and A2-7, respectively. Similar roles are played in the specification of pABLKs from A1-4. Abd-B represses the expression of Lk in A8 during the embryonic neurogenesis and in A5-8 during the larval one.
A1-8, abdominal segments; Q, quiescence; vl NB, ventrolateral NB. As there are no lineage markers for NB5-5, the number of cells and the position drawn do not correspond to reality. The expression of Ubx (green), Abd-A (blue) and Abd-B (yellow) is shown only in the ABLK.

Other similar intrincate mechanisms of segment-specific appearance of neurons mediated by Hox actions have been described in the CNS of *Drosophila* (Berger et al., 2009; Berger et al., 2005; Kannan et al., 2010; Karlsson et al., 2010; Miguel-Aliaga and Thor, 2004; Rogulja-Ortmann et al., 2008; Suska et al., 2011). In these cases Hox proteins act either at the level of the neuroectoderm, neuroblast or postmitotic progeny. In addition, their functions are performed in individual cells, cell-autonomously and at defined times during development, which warns us about the complexity of their roles and their dependence on cellular context.

We also observed that *abd-A* misexpression generated ectopic ABLKs mostly from segments T1 to A4. We propose that, as a result of of *abd-A* misexpression in NBs at late embryo, NB5-5A's entry into quiescence is prevented and precociously generates its larval lineage during embryogenesis, therefore the ectopic ABLKs detected would correspond to the pABLKs. It has been shown that the *Hox* gene *Antp* is required in NB3-3T to enter quiescence, and that both loss of *Antp* and misexpression of *abd-A* inhibited this entry (Tsuji et al., 2008). Yet, it is not known what are the mechanisms employed by the three abdominal NBs, which do not express Antp, to escape apoptosis and enter quiescence; our results indicate that Abd-A should be retained at low levels. The results presented by Prokop et al. (Prokop et al., 1998), also pointed towards this idea. They found that ectopic expression of either *Ubx* or *abd-A* reduced the numbers of eNBs in both thoracic and abdominal segments. The fact that the numbers of abdominal NBs were also decreased suggested that some NBs in the central abdomen, e.g. vm, vl and dl never expressed functional amounts of Ubx or Abd-A, so that their overexpression could trigger the elimination of these NBs. Therefore, it could be possible that upon *abd-A* misexpression eNB5-5A prematurely generates larval progeny, and eventually dies by apoptosis. Yet, it is not known how *Ubx* and *abd-A* could mediate the elimination of abdominal eNBs at the end of embryogenesis. It has been extensively studied, however, how *abd-A* promotes cell death in the postembryonic NBs of abdominal segments (Bello et al., 2003; Cenci and Gould, 2005; Maurange et al., 2008). In this case, the sole presence of Abd-A is not enough to trigger apoptosis of these NBs; but NBs need to have reached a specific temporal status, which is established by the transient embryonic/early larval pulse of Cas and by the postembryonic pulse of Svp. Again, this enhances the idea that the actions of *Hox* genes are highly dependant on a temporally-defined cellular context. Such a mechanism links the generation of neural diversity with the ending of neurogenesis, ensuring that NBs do not stop dividing until they have generated their complete set of progeny. Thus, further work will be

required to test if Ubx and Abd-A employ a similar mechanism to terminate proliferation of embryonic NBs in abdominal segments. The only example in which the role of Bx-C has been addressed in abdominal eNBs is that of NB5-6A (Karlsson et al., 2010). There, they showed that Ubx, Abd-A and their cofactors Exd and Hth promoted cell cycle exit of the NB at stage 12.

According to Abd-B, we observed that the correspondant eABLK of A8 appeared when Abd-B was removed, either with a null *Abd-B* allele or with an *Abd-B RNAi* construct from first instar larvae. These findings indicate that Abd-B represses the expression of the neuropeptide Lk but not the specification of the neuron ABLK. In addition, we detected clusters of ectopic ABLKs from segments T1 to A8 when *abd-A* was misexpressed in an *Abd-B* background. Also, we detected as many as 15 ABLKs, probably 2 by hemisegment, in adults when we removed *Abd-B* from first instar larvae. These results suggest that in normal development, Abd-B represses the expression of Lk in the eABLK of A8 during embryonic neurogenesis, whereas it does so in the pABLKs from A5-8 in the larval neurogenesis.

7.9. *Hox* genes are involved in the activation and maintenance of Lk expression.

Moreover, the fact that the removal of *Abd-B* from first instar larvae generates one extra ABLK in A8, suggests that Abd-B represses the expression of the neuropeptide Lk in an otherwise ABLK-specified neuron; thus, as soon as Abd-B repression is released, it is competent to activate Lk. This result also excludes the role of Abd-B in regulation of PCD, as it functions as a pro- or anti-apoptotic factor (Lohmann et al., 2002; Miguel-Aliaga and Thor, 2004). This reinforces the idea that the specific roles of Hox proteins in CNS development are highly context-dependent.

We also found that both Ubx and Abd-A from first instar larvae caused the loss of most ABLKs in the adult, indicating that Ubx and Abd-A are required to maintain expression of Lk.

7.10. Antp and Scr are competent to activate the ABLK fate.

Our findings about Hth indicate that it is not required to specify the ABLKs. However, we found that ectopic expression of *hth* was able to induce ABLKs in the thoracic segments, where the only Hox proteins present are Antp (T1-2) and Ubx (T3). This indicates that Antp is also competent to activate ABLK fate if enough Hth is provided.

We also detected ABLKs from T1 to A8 when *Scr* was ectopically expressed with a pan-neuronal driver. However, there are no ABLKs in parasegment 2 (posterior S2 and anterior S3), where Scr is normally expressed. These results suggests that there could be two levels for the actions of Hox genes; first the neuroectoderm where different Hox genes appear to have different functions in lineage specification; and second, in neurons, where *Scr, Antp, Ubx* and *abd-A* seem to be able to activate the ABLK fate. Further analysis will be required to know if segment-specific lineages of NB5-5 are due to changes in lineage progression, as it has been shown for NB5-6 (Karlsson et al., 2010), or in the expression of factors required for cell specification.

Unlike *Ubx* and *abd-A* misexpression, *Scr* misexpression generates ectopic ABLKs in A8 without affecting *Abd-B* expression. It has been shown that a mechanism of competition for cofactor-dependent-DNA-binding explains Hox phenotypic suppression (Noro et al., 2011). But contrary to the previously observed posterior prevalence (Gonzalez-Reyes and Morata, 1990; Mann and Hogness, 1990), in this case, Scr shows dominance over Abd-B. Further studies would be required to confirm whether the Lk enhancer is a direct target of the *Hox* genes.

8. Conclusions

1. The onset of Lk expression in the abdominal ganglion takes place at two different stages during development. At late embryonic stage it shows a fixed pattern of 7 ABLKs per hemiganglion, whereas it consists of a variable pattern of around 10 ABLKs per hemiganglion in the adult stage.

2. NB5-5A is the progenitor of all ABLKs. When it delaminates at late stage 11, it starts its temporal sequence with Pdm, it follows with Cas at stage 12, and it coexpresses Cas and Grh by stage 13. ABLKs are generated during this Cas/Grh temporal window.

3. Both ABLK and its sibling cell have the potential to differentiate into ABLKs, but are fated to die unless Notch signalling gets activated.

4. The genes *castor, grainy-head*, *nab, klumpfuss, squeeze, jumeaux, knirps* and *teashirt* are involved in the specification of ABLKs. Cas activates the expression of *nab, sqz, grh,* and *klu*, and acts both upstream and downstream these genes.

5. Sqz represses, either directly or indirectly, ABLK specification. This inhibitory activity of Sqz is blocked by Nab, possibly by forming a complex with it, and also by Klu.

6. ABLKs have two distinct origins: embryonic and postembryonic neurogenesis. Both are generated by the same progenitor NB: embryonic NB5-5A and ventrolateral postembryonic NB.

7. ABLKs specification requires the action of the Hox protein Ubx in A1, and the redundant roles of Ubx and Abd-A in A2-7 segments.

8. The ectopic expression of *abd-A* represses the entry into quiescence of NB5-5A, leading to the generation of both embryonic and postembryonic lineages in embryonic stages, without interruption.

9. Abd-B represses the expression of Lk. In particular, it inhibits ABLK fate in abdominal segment 8 during embryonic neurogenesis, whereas it does so in abdominal segments 5 to 8 during larval neurogenesis.

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10. Presentación en castellano

Resumen

El sistema nervioso central (SNC) posee mayor diversidad celular que ningún otro órgano, es por ello que se ha convertido en un excelente modelo para el estudio de la especificación celular. En este trabajo, nos hemos centrado en el estudio de los mecanismos implicados en la especificación de las neuronas leucokinérgicas abdominales (ABLKs), localizadas en la cuerda nerviosa ventral del SNC de *Drosophila,* y caracterizadas por la expresión del neuropéptido Leucokinina (Lk). Estas neuronas forman un grupo de 14 células, una por hemineurómero, en los segmentos abdominales A1-7.

Primero, mediante experimentos de colocalización de varios marcadores moleculares y análisis de linaje, hemos identificado al neuroblasto (NB) 5-5 abdominal (A) como el progenitor que da lugar a las ABLKs. Además, estas neuronas se especifican en una ventana temporal Castor/Grainy-head (Cas/Grh).

Segundo, hemos encontrado que las dos células postmitóticas, la ABLK y su célula hermana, son igualmente competentes para activar la expresión de Lk, pero están destinadas a morir por apoptosis a menos que activen la ruta de señalización de Notch y ésta inhiba el programa apoptótico.

Tercero, realizamos una búsqueda para identificar genes implicados en la especificación de las ABLKs, y encontramos a los genes *nab, klumpfuss (klu), squeeze (sqz), jumeaux (jumu), knirps (kni)* y *teashirt (tsh)*.

Asimismo, observamos que el número de ABLKs aumentaba durante los estadios pupales, alcanzando una media de 10 neuronas en adultos de cuatro días de edad. Mediante experimentos de linaje, concluímos que las ABLKs presentes en adultos correspondían a las ABLKs embrionarias (eABLKs) además de otras generadas durante la neurogénesis larvaria; a las cuales denominamos por tanto, ABLKs postembrionarias (pABLKs). Posteriormente, descubrimos que las pABLKs provenían del NB postembrionario ventrolateral (pNB vl), y concluímos que el NB5-5A embrionario y el pNB vl eran el mismo neuroblasto.

Por último, estudiamos el papel de los genes *Hox* en la definición del patrón segmental de las ABLKs, e identificamos la implicación de los genes del complejo Bithorax (Bx-C). Hemos encontrado que Ultrabithorax (Ubx) y Abdominal-A (Abd-A) se requieren de manera redundante en este proceso, mientras que Abdominal-B (Abd-B) reprime la expresión de Lk en el segmento abdominal 8 durante la neurogénesis embrionaria, y en los segmentos A5-8 durante la neurogénesis larvaria.

Introducción

La biología del desarrollo estudia los procesos por los cuales un organismo crece y se desarrolla, a partir de una única célula a un individuo adulto. En esencia, la pregunta fundamental de la biología del desarrollo consiste en comprender cómo un sistema se genera funcionalmente, cómo las distintas células que lo forman son capaces de integrar las numerosas señales que reciben y cómo se consigue diversidad celular a partir de un grupo de células progenitoras relativamente pequeño y homogéneo. El desarrollo del sistema nervioso es un buen modelo para abordar estas preguntas.

El objetivo de esta tesis es estudiar los mecanismos implicados en la especificación de un grupo de neuronas presentes en el SNC de *Drosophila*. Estas neuronas expresan el neuropéptido Lk y están presentes en los siete primeros segmentos abdominales.

El SNC de *Drosophila* se divide en cerebro, ganglio subesofágico y cuerda nerviosa ventral (CNV), la cual sería el equivalente de la médula espinal en vertebrados. Cada una de estas regiones está segmentada, así, la CNV se divide en 3 segmentos torácicos (T1-3) y 10 segmentos abdominales (A1-10). Cada uno de estos segmentos posee simetría bilateral, estando formado por dos mitades separadas por la línea media; las cuales se llaman hemisegmentos o hemineurómeros. Las células nerviosas progenitoras se denominan neuroblastos (NBs). En la CNV los NBs se dividen se manera asimétrica, renovándose y generando una célula hija de menor tamaño, llamada célula madre ganglionar (CMG). Ésta se divide una sola vez para dar lugar a dos células hijas, que pueden ser neuronas o células gliales. Hay unos 30 NBs por hemisegmento en la CNV, cada uno de estos NBs posee un linaje único caracterizado por el número y tipo celular que genera.

La identidad de cada NB está definida por la expresión combinatorial de dos grupos de genes; los genes segmentales, en el eje anteroposterior, y los genes columnares, en el eje dorsoventral. De este modo, la expresión de estos genes define un conjunto de filas y de columnas en el que cada NB posee un destino único en función de la posición que ocupa. Por otra parte, la identidad de los diferentes tipos neurales generados está regulada por la expresión secuencial de factores de transcripción llamados genes temporales, estos son *hunchback (hb), Krüppel (Kr), pdm, castor (cas)* y *grainy-head (grh).* Esto permite que los NBs puedan producir distintos tipos celulares a lo largo del desarrollo.

Además, a lo largo del eje anteroposterior, los genes *Hox* proporcionan identidad segmental. Estos codifican factores de transcripción con homeodominio y están conservados de nemátodos a vertebrados, tanto con respecto a su función como con respecto a su organización en complejos génicos. En *Drosophila* existen dos complejos de genes *Hox*, el complejo Antennapedia (Antp-C), encargado de especificar los segmentos de la cabeza y tórax anterior y el complejo Bx-C, que especifica los segmentos de tórax posterior y abdomen. Antp-C está formado por los genes *labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr)* y *Antennapedia (Antp).* El Bx-C está compuesto por los genes *Ubx, abd-A* y *Abd-B.* Asimismo, las proteínas Hox usan cofactores para aumentar su especificidad de unión a regiones de ADN. En *Drosophila* se han identificado dos*:* Extradenticle (Exd) y Homothorax (Hth).

Drosophila, al ser un insecto holometábolo, es decir, que posee una fase de desarrollo larvaria y otra pupal anterior al estadio adulto, tiene dos períodos de neurogénesis, que dan cuenta de los dos sistemas nerviosos diferentes de la larva y el adulto. De este modo, durante la neurogénesis embrionaria se genera el sistema nervioso larvario, mientras que en la neurogénesis larvaria o postembrionaria se genera la mayor parte del sistema nervioso del adulto. Estas dos neurogénesis están separadas por una fase de quiescencia, no proliferativa de los NBs. La mayoría de los NBs embrionarios de la CNV entran en quiescencia al final del primer estadio larvario, mientras que algunos mueren por apoptosis. En los segmentos torácicos, >20/30 NBs por hemisegmento entran en quiescencia, pero en los segmentos abdominales centrales (A3-7) solo lo hacen 3 NBs. Estos 3 NBs, salen de quiescencia al final del segundo estadio larvario y comienzan a proliferar de nuevo. Estos 3 NBs postembrionarios han sido identificados en función de la posición que ocupan pero no se conoce cuáles son sus

equivalentes embrionarios; se les ha denominado ventrolateral (vl), ventromedial (vm) y dorsolateral (dl), según su posición en la CNV.

El sistema leucokinérgico en larvas de tercer estadio está formado por cuatro grupos de neuronas que expresan el neuropéptido Lk. Estos son: las neuronas Lateral Horn Leucokinérgicas (LHLKs), situadas cerca del órgano "Lateral horn" en el cerebro adulto; un grupo de 2-4 células presentes en el lóbulo cerebral anterior (ALKs); dos pares de neuronas localizadas en el ganglio subesofágico (SELKs), y 7 pares de neuronas Leucokinérgicas en los siete primeros segmentos abdominales (ABLKs).

Objetivos

El objetivo de esta tesis ha sido el análisis de los mecanismos de especificación de las ABLKs. Para ello nos fijamos los siguientes objetivos concretos:

1. Análisis del patrón de expresión de la Lk en estadios embrionarios tempranos.
2. Identificación del NB progenitor de las ABLKs.
3. Identificación de los factores temporales implicados en la especificación de las ABLKs.
4. Estudio del papel de la muerte celular programada y de la ruta de señalización de Notch en la especificación de las ABLKs.
5. Búsqueda de genes que participan en la especificación de las ABLKs.
6. Análisis del patrón de expresión de las ABLKs desde tercer estadio larvario al estadio adulto.
7. Determinación del origen de las ABLKs presentes en el sistema nervioso central del adulto.
8. Análisis del papel de los genes *Hox* en la definición del patrón segmental de las ABLKs a lo largo del desarrollo.

Resultados

Primero describimos el patrón de expresión de las ABLKs en primer estadio larvario y vimos que era muy similar al ya descrito en larva 3.

A continuación, mediante experimentos de colocalización entre las ABLKs y una batería de marcadores moleculares propios de NBs, identificamos al NB5-5 como el posible NB progenitor de las ABLKs. Además, mediante un experimento de linaje marcamos toda la progenie de los NBs de la fila 5 y observamos que las ABLKs formaban parte de esta progenie, lo que reforzó nuestra hipótesis. Luego, describimos la serie de factores temporales que se expresaban en el NB5-5 y vimos que empezaba con una fase Pdm corta, luego expresaba Cas a partir del estadio 12, y finalmente coexpresaba Cas y Grh a partir del estadio 13. Posteriormente, y mediante experimentos de falta y ganancia de función, confirmamos los requerimientos de Cas y Grh en la especificación de las ABLKs.

También estudiamos el papel de la apoptosis y la ruta de Notch. Primero, mostramos que la ABLK tenía una célula hermana que moría por apoptosis. Posteriormente, descubrimos que estas dos células postmitóticas tenían la misma capacidad para diferenciarse como ABLK, pero la ruta de Notch debía activarse en una de ellas para inhibir el programa apoptótico.

En cuanto a la búsqueda de genes implicados en la especificación de las ABLKs, encontramos fenotipo de pérdida o ganancia de ABLKs en los mutantes para los siguientes genes: *nab, klu, sqz, jumu, kni* y *tsh*. Así, realizamos un análisis de interacciones genéticas, del que concluímos que Cas activa la expresión de *nab, klu, grh* y *sqz;* mientras que Sqz ejerce una represión, directa o indirecta, sobre el destino ABLK. Por su parte, Nab, posiblemente uniéndose a Sqz, es capaz de inhibir este bloqueo. La función de Klu sobre Sqz sería similar. Por el momento desconocemos el papel preciso de Kni y Tsh en la especificación de las ABLKs.

A continuación, observamos que el número de ABLK aumentaba en estadios pupales llegando a una media de 10 ABLKs por hemiganglio en adultos de cuatro días de edad. Mediante experimentos de linaje concluímos que 7 de las ABLKs observadas en el adulto eran generadas durante la neurogénesis embrionaria, y que las restantes se producían en la larvaria; a estas últimas las denominamos ABLKs postembrionarias (pABLKs). Quisimos

identificar de qué NB postembrionario provenían, así que buscamos la coincidencia en la expresión de marcadores moleculares entre estos 3 NBs y las pABLKs; determinamos que procedían del NB postembrionario ventrolateral (vl). Posteriormente, marcamos el linaje de NBs embrionarios de la fila 5, y encontramos clones, en segundo estadio larvario, en los que estaban incluídas las ABLKs embrionarias y el pNB vl. De este modo concluímos que el NB5-5A y el pNB vl eran el mismo neuroblasto.

Por último, estudiamos el papel de los genes *Hox*. Vimos que Ubx era necesario para especificar las ABLKs de segmentos A1-7, mientras que Abd-A lo era para las ABLKs de los segmentos A2-7. Además, la expresión ectópica de *abd-A* era capaz de adelantar la aparición de las pABLKs en estadios embrionarios, probablemente evitando que el NB5-5A entre en quiescencia y permitiendo así que siga proliferando. También descubrimos que tanto Ubx como Abd-A eran necesarios para el mantenimiento de la expresión de Lk a lo largo del desarrollo. Por su parte, Abd-B reprime la activación del neuropéptido Lk en la ABLK del A8 durante la neurogénesis embrionaria, y en las ABLKs de los segmentos A5-8 durante la neurogénesis larvaria. También vimos que la sobreexpresión del cofactor Hth y Scr era capaz de generar ABLKs ectópicas en segmentos torácicos.

Discusión

Aunque el tipo y número de células neurales generadas por cada NB embrionario ha sido extensamente estudiado, la asignación de una neurona concreta a un linaje específico solo se sabe en muy pocos casos. De hecho, solo se conoce parte de la progenie en 12 de los 30 NBs presentes por hemisegmento. De estos 12, se conoce el linaje completo de 3 de ellos, de los NB3-3, 5-6 y 7-3. Esto ha sido posible gracias a la existencia de marcadores de linaje. En este trabajo, hemos asignado las ABLKs al NB5-5A, sin embargo, debido a la falta de marcadores de linaje no ha sido posible identificar por completo todas las células generadas.

No todos los NBs expresan la secuencia completa de factores de transcripción, hay algunos que empiezan expresando Cas. En nuestro caso, el NB5-5A comienza la serie con Pdm, además hemos detectado que las ABLKs expresan *seven-up(svp)-lacZ*, por lo que muy probablemente el NB5-5A exprese Svp en estadios embrionarios tardíos.

Por tanto, teniendo en cuenta todos los datos presentados, proponemos un modelo en el que las ABLKs serían generadas por el NB5-5A, expresando éste los genes *cas, nab, sqz, klu* y *grh*. Cas estaría actuando por encima de *nab, sqz, klu* y *grh*, ya que activa su expresión, pero también por debajo de los mismos, según apuntan los resultados de rescates cruzados realizados.

Se han descrito otros casos en los que la diferenciación terminal de neuronas peptidérgicas está retrasada, es decir, el nacimiento de la neurona y la expresión del neuropéptido en la misma ocurre en diferentes momentos del desarrollo. Para el caso de las neuronas que expresan el neuropéptido CCAP (Crustacean cardioactive peptide) se ha demostrado que la señalización por ecdisona es el desencadenante de esta diferenciación terminal. En nuestro caso, las pABLKs son generadas en estadios larvarios pero no se diferencian completamente, i.e. expresión de Lk, hasta estadios pupales.

En cuanto al papel de los genes Hox en la especificación de destinos neurales en el sistema nervioso, se ha descrito que éstos pueden actuar a nivel de neuroectodermo, neuroblasto y células postmitóticas; desarrollando sus funciones de manera celular autónoma y siendo altamente dependientes de contexto.

Conclusiones

1. El inicio de la expresión de Lk en la CNV ocurre en dos estadios diferentes del desarrollo. En estadios embrionarios tardíos, el patrón de expresión de ABLKs está formado por un número fijo de 7 neuronas por hemiganglio, mientras que en estadios adultos consiste en un patrón variable, alrededor de 10 ABLKs, por hemiganglio.

2. El NB5-5A es el neuroblasto progenitor de todas las ABLKs. Cuando delamina en estadio 11 tardío, empieza su secuencia temporal expresando Pdm, luego sigue una ventana de expresión de Cas en estadio 12, y coexpresión de Cas y Grh a partir del estadio 13. Las ABLKs son generadas en esta ventana Cas/Grh.

3. Tanto las ABLKs como sus células hermanas tienen la capacidad de diferenciarse como ABLKs, pero están destinadas a morir a menos que se active la ruta de señalización de Notch y ésta reprima el programa apoptótico.

4. Los genes *castor, grainy-head, nab, klumpfuss, squeeze, jumeaux, knirps* y *teashirt* están implicados en la especificación de las ABLKs. Cas activa la expresión de *nab, sqz, grh* y *klu*, y actúa tanto por encima y por debajo de ellos.

5. Sqz reprime directa o indirectamente el destino ABLK. Esta actividad represora es bloqueada por Nab, probablemente formando un complejo con Sqz, y por Klu.

6. Las ABLKs tienen dos orígenes distintos: a partir de la neurogénesis embrionaria y postembrionaria. Ambas son generadas por el mismo neuroblasto progenitor: el NB5-5A embrionario y el NB postembrionario ventrolateral.

7. La especificación de las ABLKs requiere de la acción de las proteínas Hox; Ubx en A1 y de manera redundante, de Ubx y Abd-A en los segmentos A2-7.

8. La sobreexpresión de *abd-A* reprime la entrada en quiescencia del NB5-5A, haciendo que el programa de desarrollo de este NB, que normalmente ocurre en embrión y larva, se produzca sin interrupción.

9. Abd-B reprime la expresión de Lk. En concreto, inhibe el destino ABLK en el segmento abdominal 8 durante la neurogénesis embrionaria, y en los segmentos A5-8 en la neurogénesis larvaria.

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12. Appendix I

Supplemental table 1: List of mutants with mild or no phenotype for ABLKs.

Name of gene	Genotype	ABLK cells mean±standard deviation (n)
abnormal spindle	*asp [1]*	χ=7.00±0.00 (7)
abrupt	*ab [1D]*	χ=7.00±0.00 (16)
apterous	*ap [P44]*	χ=7.00±0.00 (16)
apointed	*apt [k15608]*	χ=7.00±0.00 (22)
Arrowhead	*Awh [63Ea-G14]*	χ=7.00±0.00 (11)
buttonhead-gal4	*btd-Gal4*	χ=7.00±0.00 (14)
capodimonte	*cod [2]*	χ=6.89±0.32 (18)
capricious	*caps [65.2]*	χ=6.70±0.63 (23)
Chip	*Chip [e5.5]*	χ=6.71±0.59* (32)
Chip	*Chip [g96.1]*	χ=7.28±1.49 (18)
charlatan	*Df(2R) Exel 7135*	χ=7.09±0.29 (23)
collier	*col[1]*	χ=7.00±0.00 (20)
crooked legs	*crol [04418]*	χ=7.00±0.00 (7)
Cyclin-AMP response element binding protein A	*Df(3L) BSC558*	χ=7.00±0.00 (15)
dachshund	*dac [4]*	χ=7.00±0.00 (6)
daughterless	*da [1]*	χ=6.63±0.56* (30)
dissatisfaction	*dsf [f00109]*	χ=7.00±0.00 (14)
DP transcription factor	*Df(2R) BSC272*	χ=7.00±0.00 (29)
Ecdysone receptor	*EcR [Q50st]/ [k06210]*	χ=7.00±0.00 (19)
Ecdysone receptor	*EcR [Q50st]/ [31]*	χ=7.00±0.00 (25)
Ecdysone receptor	*EcR [112]*	χ=7.00±0.00 (17)
elbow noc	*elB [3.3.1] noc [delta 64]*	χ=6.83±0.38 (18)
empty spiracles	*ems [1]*	χ=7.00±0.00 (13)
escargot	*esg [35Ce-1]*	χ=6.30±0.95 (10)
escargot	*esg [35Ce-1]/[G66]*	χ=7.00±0.00 (10)
espinas	*esn [f00447]/Df(2R) Exel 6283*	χ=7.00±0.00 (20)
Ets at 98B	*Df(3R) BSC 499*	χ=6. 72±1.24 (46)
eyes absent	*eya [cli-IID]*	χ=6.95±0.31 (41)
eyeless	*eyeless [2]*	χ=7.00±0.00 (21)
eyegone	*eyegone [SA2]*	χ=7.00±0.29 (25)
Fer3	*Df(3R)Exel8154/Df(3R)Exel7309*	χ=6.80±0.61 (50)
forkhead	*fkh [1]*	χ=6.93±0.27 (27)
forkhead domain 96Cb	*Df(3R)BSC493/Ff(3R)BSC679*	χ=7.00±0.00 (21)
fruitless	*Df(3R)BSC509/Df(3R)Exel6179*	χ=7.00±0.00 (24)
fushi tarazu	*ftz [5]/[13]*	χ=7.00±0.00 (14)
germ-cell expressed bHLH-PAS	*Df(1) ED7294*	χ=6.93±0.27 (14)
gemini	*Df(2R)BSC350/Df(2R)BSC303*	χ=6.91±0.68 (22)
grain	*grn [7L]*	χ=6.88±0.64 (8)
giant	*gt [E6]*	χ=6.95±0.23 (19)
extra-extra	*exex[KK30]*	χ=6.97±0.18 (31)

heartless	*htl [AB42]*	χ=6.50±0.67 (12)
homeobrain	*Df(2R)Exel 6071/Df(2R)Exel 7166*	χ=7.00±0.00 (30)
Hairy/E(spl)-related with YRPW motif	*Df(2R) Exel 6055/Df(2R)BSC266*	χ=7.00±0.00 (16)
huckebein	*hkb [2]/[A321R1]*	χ=6.73±0.65 (11)
Heat shock factor	*Hsf [1]*	χ=7.00±0.00 (20)
jing	*Jing [01094]/ [22F3]*	χ=7.00±0.00 (19)
ken and barbie	*ken [02970]*	χ=7.00±0.00 (25)
molting defective	*mld[92]*	χ=6.81±0.75 (21)
molting defective	*mld[47]*	χ=6.89±0.47 (18)
Mothers against dpp	*elav-Gal4> UAS-mad RNAi*	χ=6.97±0.18 (30)
Nkx6	*Nkx6 [D25]*	χ=7.00±0.00 (18)
odd-skipped	*odd [01683]*	χ=7.00±0.00 (12)
osa	*osa [2]*	χ=6.80±0.63 (11)
pannier	*pnr [v1]*	χ=6.56±0.81 (16)
pannier	*pnr [vx1]*	χ=6.87±0.52 (15)
pannier	*pnr [vx6]*	χ=6.61±0.92 (38)
reaper	*rpr [XR38]*	χ=7.00±0.34 (18)
rotund	*rn [20]*	χ=6.96±0.20 (26)
retained	*rtn [1]*	χ=7.00±0.00 (23)
shuttle craft	*stc [05441]*	χ=6.67±0.88 (30)
sloppy paired 1	*slp 1 [2]*	χ=6.43±0.68* (30)
sloppy paired 1	*slp 1 [1]/[2]*	χ=6.75±0.52 (28)
tailup	*tup [1]*	χ=7.00±0.00 (15)
twin of eyeless	*toy [hdl]*	χ=6.63±0.41* (46)
tailless	*tll [1]/[l49]*	χ=6.97±0.18 (32)
vein	*vn [10567]*	χ=6.69±0.48 (13)
Zn finger domain 1	*zfh1[00865]*	χ=6.91±0.29 (34)
Zn finger domain 2	*zfh2 [LP30]-Gal4>UAS-zfh2-RNAi*	χ=6.60±0.55 (5)

All data presented as mean ± standard deviation (n: number of hemiganglia scored).
All statistics performed as two-tailed t-test assuming equal variance.
p-value<0.01 compared to *wild-type*.
* : there was significant difference compared to *wild-type*.

Supplemental table 2: List of Zinn kit deficiencies analyzed.

Symbol	Deleted segment	ABLK cells mean±standard deviation (n)
Df(2L)net-PMF	21A1;21B7--8	χ=7.00±0.00 (12)
Df(2L)BSC106	21B7;21C2	χ=7.00±0.00 (22)
Df(2L)BSC4	21B7--C1;21C2--3	χ=2.57±1.78* (56)
Df(2L)BSC16	21C3--4;21C6--8	ND
Df(2L)BSC37	22D2--3;22F1--2	χ=6.94±0.24 (18)
Df(2L)dpp[d14]	22E4--F2;22F3--23A1	χ=7.00±0.00 (18)
Df(2L)JS17	23C1--2;23E1--2	χ=0.00±0.00* (18)
Df(2L)BSC28	23C5--D1;23E2	χ=6.83±0.47 (29)
Df(2L)BSC31	23E5;23F4--5	χ=6.78±0.65 (18)
Df(2L)ED250	24F4;25A7	χ=7.00±0.00 (21)
Df(2L)BSC109	25C4;25C8	χ=7.00±0.00 (21)
Df(2L)Exel6011	25C8;25D5	ND
Df(2L)BSC169	25E5;25F3	χ=3.40±3.43* (27)
Df(2L)Exel6013	25F2;25F5	χ=6.78±0.65 (18)
Df(2L)ED347	25F5;26B5	χ=7.00±0.00 (26)
Df(2L)Exel6015	26B5;26B11--C1	χ=6.83±0.38 (24)
Df(2L)Exel9038	26C2;26C3	χ=7.00±0.00 (7)
Df(2L)Dwee1-W05	27C2--3;27C4--5	χ=7.00±0.00 (12)
Df(2L)Exel7029	27C4;27D4	χ=0.00±0.00* (18)
Df(2L)Exel7031	27F2;28A3	χ=7.00±0.00 (34)
Df(2L)ED499	27F4;28C4	χ=6.92±0.28 (25)
Df(2L)Trf-C6R31	28D2--28E1;28E5--?	χ=1.50±2.13* (50)
Df(2L)BSC111	28F5;29B1	χ=7.00±0.00 (7)
Df(2L)Exel7038	29C4;29D5	χ=7.00±0.00 (29)
Df(2L)BSC215	29D3;29E1	χ=6.58±0.58* (26)
Df(2L)BSC143	31B1;31D9	χ=6.78±0.44 (9)
Df(2L)Exel7048	31E3;31F5	χ=3.57±1.73*(23)
Df(2L)ED746	31F4;32A5	χ=0.5±0.92*(28)
Df(2L)BSC36	32D1;32D4--E1	χ=6.81±0.49 (26)
Df(2L)FCK-20	32D1;32F1--3	χ=7.00±0.00(19)
Df(2L)TE35BC-24	35B4--6;35F1--7	χ=6.44±0.89 (16)
Df(2L)Exel6038	35D6;35E2	χ=7.00±0.00 (24)
Df(2L)Exel7066	36A1;36A12	χ=6.95±0.22 (21)
Df(2L)Exel8036	36B1;36C9	χ=6.69±0.58* (36)
Df(2L)BSC148	36C8;36E3	χ=6.60±0.58* (25)
Df(2L)BSC256	36E3;36F2	χ=7.00±0.00 (34)
Df(2L)Exel6045	38A3;38A7	χ=7.00±0.00 (23)
Df(2L)ED1317	38D1;38F5	χ=6.61±0.80* (36)
Df(2L)Exel7080	38F3;39A2	ND
Df(2L)Exel6047	39A2;39B4	ND

Df(2L)Exel6048	39B4;39C2	χ=7.00±0.00 (8)
Df(2L)ED1466	39E3;40A5	χ=7.00±0.00 (33)
Df(2L)Exel6049	40A5;40D3	χ=0.00±0.00* (20)
Df(2L)BSC151	40A5;40E5	χ=0.00±0.00* (44)
Df(2L)C'	h35;h38L	χ=6.95±0.22 (20)
Df(2R)Nipped-D	h46;41C1--6>	χ=6.75±0.45 (16)
Df(2R)Exel7092	43E5;43E12	χ=6.95±0.21 (22)
Df(2R)w45-30n	45A6--7;45E2--3	ND
Df(2R)BSC132	45F6;46B4	χ=7.00±0.00 (24)
Df(2R)BSC303	46E1;46F3	χ=6.68±0.67 (19)
Df(2R)BSC281	46F1;47A9	χ=7.00±0.00 (22)
Df(2R)Exel7124	49F10;50A1	ND
Df(2R)Exel7131	50E4;50F6	χ=6.96±0.21 (51)
Df(2R)Exel7135	51E2;51E11	χ=3.85±2.74* (20)
Df(2R)Exel6063	52F6;53C4	χ=7.00±0.00 (20)
Df(2R)Exel7142	53B1;53C4	χ=5.51±2.03* (47)
Df(2R)BSC161	54B2;54B17	χ=6.88±0.33 (17)
Df(2R)BSC355	54B16;54C3	χ=6.92±0.28 (25)
Df(2R)Exel7150	54E1;54E9	χ=6.91±0.30 (32)
Df(2R)ED3923	57F6;57F10	χ=0.00±0.00* (22)
Df(2R)Exel6082	60C4;60C7	χ=6.92±0.28 (25)
Df(2R)Px2	60C4--60C6;60D9	χ=6.85±0.37 (20)

All data presented as mean ± standard deviation (n: number of hemiganglia scored).
All statistics performed as two-tailed t-test assuming equal variance.
p-value<0.01 compared to *wild-type*.
* : there was significant difference compared to *wild-type*.
ND: not determined (embryos died before 18h. AEL).

13. Appendix II